EVALUATION OF THE SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVITIS USING NEXT GENERATION SEQUENCING TECHNOLOGY

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EVALUATION OF THE SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVITIS USING NEXT GENERATION SEQUENCING TECHNOLOGY"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. Swarna Alamelu, M.D.S.**, Reader, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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CERTIFICATE

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This dissertation is submitted to **THE TAMILNADU DR.MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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CONTENTS

LIST OF ABBREVATIONS

ABBREVATION	EXPANSION
PSD	Polymicrobial Synergy and Dysbiosis
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
Rrna	Ribosomal Ribonucleic acid
NGS	Next Generation Sequencing
PCR	Polymerase Chain Reaction
RT-PCR	Real Time Polymerase Chain Reaction
CFU	Colony Forming Unit
OUT	Operational Taxonomic Unit
SoLiD	Supported Oligonucleotide Ligation and Detection
BLAST	Basic Local Alignment Search Tool
MSR	Mi Seq Reporter Software
HOMD	Human Oral Microbiome Database
HOMIM	Human Oral Microbiome Identification Microarray

LIST OF TABLES

LIST OF GRAPHS

LIST OF FIGURES

Introduction

INTRODUCTION

Periodontal disease is a chronic inflammatory condition that results in destruction of the attachment apparatus of the tooth eventually leading to the loss of teeth. Although the plaque microbiota is said to initiate the disease process, it is host immune-inflammatory response that does the tissue damage. It is now widely accepted that the bacterial communities in the biofilm undergo a shift or what is known as dysbiosis, in which the presence of the disease associated species may exacerbate the inflammatory reaction to the commensal bacteria.

A microbiota is an ecological community of commensal, symbiotic and pathogenic organisms found in all multicellular organisms studied till date ranging from plants to the highest order of the animal kingdom. They have been found to be crucial for the immunologic, hormonal and metabolic homeostasis of the host[5].

The salient clinical features of periodontal disease are gingival inflammation, formation of periodontal pockets and gingival recession. Gingivitis is a reversible form of periodontal disease that is characterised by gingival inflammation in response to plaque biofilm. In susceptible individuals untreated gingivitis may progress to periodontitis.

Ever since the essential role of plaque in gingivitis was first demonstrated by the famous "Experimental gingivitis model" of **Loe**, there

has been a huge leap in our understanding of the microbial profile of the plaque biofilm and its association to periodontal disease[79].

Newer culture independent molecular methods such as those based on cloning and Sanger sequencing of the 16s rRNA have greatly expanded our knowledge of the microbial communities in the biofilm. The emergence of advanced genomic technologies like the high throughput sequencing , also known as the deep sequencing of the 16s rRNA gene or the next generation sequencing is a landmark in the development of sequencing techniques and has immensely contributed to the study of the microbiome.

With all these findings, it is now known that the PSD model might be involved in the initiation of chronic periodontitis, with the emergence of the key stone pathogen concept[48]. There is still a paucity of literature regarding the knowledge of the microflora in the entire spectrum of periodontal disease, ranging from health to gingivitis and its transition to periodontitis.

Also, it is now known that microbiome varies from one individual to another and there is a considerable difference among population and ethnic group[104]. Racial and population based difference may be influenced by dietary and lifestyle pattern. The diversity among the microbiome of individuals is immense compared to genomic variation: individual humans are about 99.9% identical to one another in terms of their host genome but can be 80-90% different from one another in terms of the microbiome of their oral cavity or

gut[21,153]. These findings suggest that employing the variation contained within the microbiome will be much more fruitful in personalized medicine, than approaches that target the relatively constant host genome.

The microbiome of the subgingival environment in various periodontal conditions has been studied in the past but there is a dearth of information regarding the microbial profile in gingivitis.

Hence this study was undertaken as a first of its kind to evaluate the subgingival microbiome in gingivitis and periodontal health in a South Indian population.

Aim and Objectives

AIM AND OBJECTIVES

- To evaluate subgingival microbiome from plaque samples of periodontally healthy individuals and in patients with gingivitis using NGS technology.
- To compare the subgingival microbiome of gingivitis with that of periodontal health.

Review of Literature

REVIEW OF LITERATURE

Oral microbiome:

The human microbiota consists of the 10-100 trillion symbiotic microbial cells harboured by each person, primarily bacteria in the gut[148]. The human body is composed not only of human cells, but is occupied by bacteria, archaea, fungi and viruses; this ensemble of organisms (microbiota) and their expressed genes are termed the microbiome. Despite their small size, the human-associated microbiota have a genetic composition that is at least two orders of magnitude greater than the human genome and it outnumbers the cells of human host; the bacterial component alone is estimated to be equal in number to that of human cells.

Different areas of our body have distinct microbial compositions that are reflective of that microenvironment. Mucosal surfaces, including the mouth, intestines, vagina and lung also provide niche environments in which different microbiomes flourish. In a healthy state, these microbiomes form symbiotic relationships with the host; the microbes are within a stable and nourishing environment, whereas the host benefits in terms of metabolism, immune system priming, and protection from other more pathogenic organisms.

While there is variability in the microbes inhabiting different individuals, the microbiomes between individuals have shared core functionalities that are relevant to the symbiotic relationship that exists between the microbiome and its host. A disturbance of the levels and function of the microbiome, termed dysbiosis, can lead to systemic problems with serious impacts on human health.

The term microbiome was coined by **Joshua Lederberg** in 2001. Microbiome is defined as the totality of the micro-organisms and their collective genetic material present in the human body or oral cavity[85]. This term has been adopted by the Human Microbiome Project and considered as the favoured nomenclature to define the complex oral bacterial community, their genetic elements and environmental interactions, which may be involved in disease[31].

Studies of the diversity of the human microbiome started with **Antonie van Leewenhook**, who, as early as the 1680s, had compared his oral and fecal microbiota. He noted the striking differences in microbes between these two habitats and also between samples from individuals in states of health and disease in both of these sites[36].

Thus, studies of the profound differences in microbes at different body sites, and between health and disease are as old as microbiology itself. What is new today is not the ability to observe these obvious differences, but rather the ability to use powerful molecular techniques to gain insight into why these

differences exist, and to understand how we can affect transformations from one state to another[1,33,95,144].

Turnbaugh et al [147] classified human microbiome into 2 types such as core microbiome and a variable microbiome. Core microbiome is one in which it is common among the individuals in healthy condition in different sites consisting of a predominant species[134,147,157]. Variable microbiome is one in which it is particular to the individual which may be due to lifestyle which may be unique, phenotypic and genotypic features. Oral microbiome of an individual is similar to that of their fingerprint as there are difference in the species and strain level of microbiome[30].

Paster et al[106] in his study had found that the estimated number of bacterial species in oral cavity was about 700. Periodontal disease condition becomes nutritionally richer environment for bacteria, thus higher bacterial diversity can be identified under diseased conditions but there may be inter-individual differences in the disease group which cannot be ruled out[119]. Gingivitis or periodontitis is accompanied by a shift in the oral bacterial community, structure and composition [2,20,45,61,77].

Gingivitis:

Albandar [4] stated that gingivitis which may lead to periodontitis, is the major causative factor for tooth loss. Gingival inflammation, gingival

bleeding, and increased pocket depth are the characteristics of periodontal disease[9]. **Moore et al.**[94] defined gingivitis as a non-destructive periodontal disease of the gingiva surrounding the bacterial biofilm.

Berchier et al.[16] stated that the effects of gingivitis are reversible which can be improved by maintaining proper oral hygiene. **Dietrich et al**[35] stated that when left untreated, gingivitis may progress into periodontitis where as other studies have stated that gingivitis does not always develop into periodontitis[6,145]. **Page et al.**[103] added that periodontitis is always preceded by gingivitis.

The American Academy of Periodontology[140] defined gingivitis as a non-destructive disease that occurs around the teeth. Bacterial biofilms that are attached to the tooth contribute to the most common form of gingivitis known as plaque-induced gingivitis, which acts to initiate the body's host response thereby leading to the gingival tissues destruction resulting in the destruction of the periodontal attachment apparatus[111].

Gingivitis is a reversible form of periodontal disease characterized by inflammation of the gingivae in response to a mature dental plaque biofilm. In susceptible individuals persistent gingivitis may lead to chronic periodontitis, which causes irreversible destruction of periodontal tissue[117].

Even though the composition of subgingival plaque is primarily associated with gingivitis, as quantified by bleeding on probing, rather than the presence or absence of periodontitis, the presence of periodontitis has detectable associations with subgingival microbiota that are unrelated to gingivitis[158]. In particular, the differences in co-occurrence patterns of taxa between women with and without periodontitis support a more complex etoiology of disease than a simple progression from health through gingivitis to periodontitis[120].

Gingivitis and periodontitis were associated with higher microbial community richness and Shannon indexes, and this association remained after adjustment for demographic factors, including age, body mass index (BMI), and socioeconomic status[159]. This finding is consistent with previous research by various authors who proposed, with higher diversity meaning that, in periodontal disease, the oral microbiota is added rather than existing taxa undergoing replacement[22,61]. This may correspond to primary ecological succession in a new environmental niche, as suggested by **Abusleme et al**[2].

Liam Shaw et al[120]found that many taxa were associated with gingivitis and periodontitis. The abundance of the majority of these taxa increased with gingivitis severity, and this pattern was not influenced by the presence of periodontitis. .It would appear that relative bacterial abundances alone are insufficient to explain the presence of disease, which is consistent with a requirement for other factors, such as the host inflammatory response,

to cause disease. He identified distinct signals associated with gingivitis and periodontitis in subgingival plaque, with a dominant contribution from gingivitis. Network analysis of observed co-occurrence patterns was consistent with the role of bridging bacteria like F. nucleatum and F. alocis in the co-aggregation of periodontal biofilms prior to entrance into subgingival regions. Although some periodontitis-associated bacteria were also associated with gingivitis, the major change with periodontitis is in the network of co-occurrences.

Gingivitis sets the stage for periodontitis to develop by providing an environment where periodontitis-associated taxa can increase in abundance and co-aggregate into pathogenic biofilms that may then penetrate to subgingival regions[10,17].

The open contacts between teeth, gingival grooves, bulky and overhanging restorative margins, claps of removable partial dentures and calculus which serves for plaque accumulation are known to be plaque retentive factors[152]. The various degradative enzymes and toxins such as lipopolysaccharide (LPS), other endotoxin or lipoteichoic acid (LTA) are produced by bacteria which may lead to inflammatory response in the periodontal structures[154].

The initial stages of plaque is characterised by gram-positive cocci and rods while the latter by an increase in gram-negative rods, fusiforms, filaments, spirilla and spirochetes as the plaque matures which leads to

periodontal disease. The association of plaque to gingivitis was confirmed by the study done by **Loe** on the Srilankan tea labourers which popularly came to know as the "Experimental Gingivitis" model[80].

Experimental gingivitis:

Loe et al.[79] described the development of gingivitis exclusively in a system of model known as experimental gingivitis.

Gram-positive rods, gram-positive cocci and gram negative cocci were the initial microbiota of experimental gingivitis. Gram-negative rods and filaments, spirochaetal and motile microorganisms increase in number resulting in inflammatory changes leading to gingivitis.

He founded that 56% gram-positive bacteria, 44% gram negative bacteria are present in plaque induced gingivitis; which also includes 59% facultative organism and 41% anaerobic organisms. Most predominant gram-positive organisms includes

S.sanguis, S.mitis, S.intermedius, S.oralis, A.viscosus, A.naeslundii, and P.micros. The most predominant gram-negative organisms includes F.nucleatum, P.intermedia, and V.parvula, Haemophilus , Capnocytophaga and Campylobacter species.

Experimental gingivitis in man:

Theilade et al[141] in an experiment carried out in 11 subjects who had excellent oral hygiene and healthy gingiva wherein they developed accumulations of plaque and generalized gingivitis after 9 to 21 days without oral hygiene. It was found that the rate of plaque accumulation was correlated with the rate of development of gingivitis. Gram-positive cocci and rods were present initially when the teeth were clean and healthy gingiva.

During the first two days without oral hygiene, there developed the first phase of plaque. Proliferation of gram-positive cocci and rods were seen along with an addition of about 20 to 30% gram-negative cocci and rods. Fusobacteria and filaments began to appear and increased about 7% of the total flora during the second phase that is about 1-4 days.

Spirilla and spirochetes contributed for about 2% of the total flora during the third phase; that is after 4-9 days. The composition of the plaque correlated with the condition of the gingiva in certain areas which helped in the clinical diagnosis of the gingivitis which in turn correlates with the same time as this complex flora begin to colonize but the sub-clinical inflammation started at the first phases of plaque development.

After this experiment the subjects were advised to start the oral hygiene measures and it was noted that within 1 to 2 days, plaque began to disappear indicating that the gingivitis began to reduce one day after the

removal of plaque. Plaque index were taken after 7–11 days which returned to the baseline value.

A was study done by **Loe et al** [80] from 1970 to 1986 among the tea labourers in Srilanka which included 480 males of age group ranging from 14 – 46 years, who did not perform any conventional oral hygiene measures and were able to displace large aggregates of plaque and calculus on their teeth. They were divided into 3 groups: RP group: about 8% of individual had rapid progression of periodontal disease, MP group: about 81% of individuals had moderate progression and NP group: about ~11 % of individuals had no progression of periodontal disease beyond gingivitis. In RP group the mean loss of attachment was ~9 mm, the MP group had ~4 mm and the NP group had less than 1 mm loss of attachment at 35 years of age. The mean loss of attachment in the RP group was ~13 mm and the MP group ~7 mm at 45 years of age. The annual rate of destruction in the RP group varied between 0.1 and 1.0 mm, in the MP group between 0.05 and 0.5 mm, and in the NP group between 0.05 and 0.09 mm. In the RP group, tooth loss already occurred at 20 years of age and increased throughout the next 25 years. At 35 years of age, 12 teeth had been lost, at 40 years of age 20 teeth were missing and at 45 all teeth were lost. In the MP groups, tooth mortality started after 30 years of age and increased throughout the decade. At 45 years of age, the mean loss of teeth in this group was 7 teeth. The NP group essentially showed no tooth loss. This study proves the role of plaque in the periodontal disease.

Role of plaque in periodontal disease:

The etiological role of plaque in periodontal disease, the initiation and the progression of periodontal diseases was clearly explained by Loe's experimental gingivitis but with the time, the ideas about how a shift from oral health to disease due to dental plaque have been changed[79].

Non-specific Plaque hypothesis:

Investigation done by **Miller**[92] opened a window to the non-specific plaque hypothesis. They postulated that it was the quantity of plaque that determines the pathogenicity without discriminating between the levels of virulence of bacteria. According to **Theilade**[142], when plaque content with its toxins and breakdown products exceeded the capacity of host response, disease occurs. He also stated that all bacteria in plaque contribute to the virulence of the microflora by having a role in either colonisation, evasion of the defence mechanism, and/or provocation of inflammation and tissue destruction.

Page RC[103] stated that however this hypothesis does not suit for the development of periodontitis, whereas it is applicable only for the development of gingivitis since periodontitis is a multifactorial disease. The main lack of this hypothesis is that it failed to prove that why all gingivitis not progress to periodontitis. **Socransky et al**[133] pointed out that this hypothesis failed to explain as to why in some subjects aggressive and advanced forms of

periodontitis had little plaque whereas in some subjects with mild periodontitis had increased amount of plaque and also site specificity of the disease is inconsistent with the concept that all plaque is equally pathogenic.

Specific Plaque Hypothesis:

The specific plaque hypothesis, proposed by **Walter J. Loesche**[83], stated that the quality and not the quantity of plaque mattered as only certain microorganisms in plaque were thought to be pathogenic. When these specific bacteria increased in number, virulence factor released by them would lead to periodontal diseases. For instance in localized aggressive periodontitis, Aggregatibactoractinomycetacomitans is found to be the specific pathogen.[98,129]

Following the development and maturation of dental plaque, with increase in probing depth, oral microbial flora specifically changes from gram-positive aerobic species to gram-negative anaerobic species[89,131]. **Socransky** and **Haffajee**[132] identified specific microbial groups with dental plaque. Six inter-related groups were reported. The early colonizer consists of yellow, green and purple complexes. These complexes help in the colonization of orange and red complexes. The red complex organisms are associated with periodontitis which includes Tannerella forsythia, Porphyromonas gingivalis and Treponema denticola. The main disadvantage of this hypothesis is that it failed to explain why Porphyromonas gingivalis, Tannerella forsythia which

are considered to be the main putative periodontal pathogens are mostly present in healthy periodontium.

Ecological Plaque Hypothesis:

Philip D. Marsh[89] had put forth the Ecological plaque hypothesis. This hypothesis stated that the disease is the result of an imbalance in the total microflora due to ecological stress, resulting in an enrichment of some oral pathogens or disease related organisms. More specifically, this hypothesis proposes that the nonspecific accumulation of plaque leads to inflammation within the gingival tissues and to the development of gingivitis. This leads to environmental changes within the gingival sulcus, which in turn favour the growth of gram-negative and proteolytic species of bacteria. These changes lead to further inflammatory and immune mediated tissue changes and tissue destruction, culminating in a predominance of periodontal pathogens and a greater degree of tissue damage.

Keystone Pathogen Hypothesis:

Hajishengallis et al.[48]stated that the quantity of normal microbiota and their composition can be changed by some low-abundance microbial pathogens there by causing inflammatory disease. **Socransky et al**.[131] stated that these keystone pathogens can be detected in higher numbers when the disease reaches the advanced stage.

Polymicrobial Synergy and Dysbiosis Model:

Hajishengallis et al[48] had described Polymicrobial Synergy and Dysbiosis (PSD) model of pathogenesis. He founded that periodontitis is initiated by a broadly based dysbiotic, synergistic microbiota as against the traditional view that it is caused by a single or several pathogens. Thus the host-microbe homeostasis is altered by this dysbiotic, synergistic microbes and there by leading to a transition of chronic inflammatory state. Thus the disease progression is caused by whole microbial community.

The interaction between the micro-organisms present in the plaque and immune responses results in tissue destruction that includes both the loss of connective tissue and alveolar bone[70].

Microbiological Methods / Assays in Periodontal Diagnosis:

It has been estimated, however, that approximately half of the bacteria found in the oral cavity have not been, or cannot be, cultivated in the laboratory[151,23,26,139]. Therefore, culture studies alone could not provide a comprehensive description of the microbiota in experimental gingivitis.

The early knowledge of the microbial composition of dental plaque in gingivitis is based largely on microscopy and cultural methods, which do not provide a comprehensive description of oral microbial communities.

Close ended techniques:

Culture:

Traditionally identification of the species in any given sample was achieved by growing it in vitro on suitable media. There are 2 types of media for culturing, which are the selective media and non-selective media. **Samaranayake**[115] founded that growth of a broad spectrum of organisms can be enhanced by blood agar which is a common non-selective medium. Laboratory culturing under special conditions and using a range of media has allowed isolation of a diverse range of bacteria. However, it is well recognized that the main drawback of this method is its narrow spectrum. Various authors founded that about 50% to 60% of distinct bacterial phyla in oral cavity are still non cultivable[62,126,149,136]. Moreover, the culture-dependent technique is expensive, sensitive and needs a highly skilled individual.

Siqueria et al.[125] stated that there is a growing need for developing improved methods to cultivate and characterize the as-yet-uncultivated portion of the oral microbiome so as to unravel its role in health and disease. Theoretically, all bacteria can grow under proper nutritional and physicochemical conditions[24]. However, development of new improved culture media is still a challenging goal and is mainly due to the highly diverse microbial community present with each member having different nutritional requirements. Certain authors has recently suggested a list of recommendations in order to cultivate the yet-uncultivated bacteria, such as

the use of culture media with little or no added nutrients and addition of specific growth factors in the culture media[126,143].

A very interesting strategy to ensure the availability of natural growth factors is to perform incubation in the natural environment using special devices [43,59,127,137] such as a diffusion chamber or a hollow fibre membrane chamber which allow diffusion of important growth factors from a natural environment to the culture via a special membrane[8,19,50,74,122].

Though considered gold standard for identification and analysis of bacteria, bacterial cell culture has its own limitations. Only viable bacteria will grow, and stringent transport and storage conditions will be needed to identify those bacteria that are 'cultivable'[18,37]. Those that are not cultivable would remain unidentified. This could be due to the fact that many species in the oral cavity are fastidious and require the presence of other organisms and very specific growth conditions. If those conditions, that could even be unknown and unexplored, are not provided, then the bacterial species that are the most fastidious will simply not grow in culture and will remain unidentified. Bacterial identification using newer molecular methods will surpass the limitations associated with culture based methods for identifying bacteria[57,99,110].

Immunologic and enzymatic assays:

Accurate detection of targeted species can be achieved by the antibody-based detection systems. Raising of antibodies to each species of interest needs growing the organism in culture before inoculating an animal and raising antibodies to the bacterial antigens. **Wolff, L.F et al.**[155], **Loesche et al.**[82] used this technique to identify the changes in the pathogens either naturally or in response to treatment in healthy and disease population.

Limitation of immunological assays is that the target organism has to be cultured to raise antibodies against it. This makes this method useful only for cultivated species[38,93]. The antibodies cross reactivity can be tested only on cultivated species and cannot be done on uncultivated or unknown species.

DNA – DNA hybridization or checkerboard:

DNA-DNA hybridization detects bacteria with the help of a labeled genomic DNA that is attached to nylon membranes based on hybridization of target microbes. In periodontal health and disease including the response to the treatment, the various levels of microbes have been studied with this technique[40,44,47,81,133,156]. They used data from population based studies and grouped 40 species that were found in clusters or 'complexes' by comparing the levels of these species in health and disease. Three species, Porphyromonas gingivalis, Tannerella forsythia (Bacteroides forsythus) and Treponema denticola, were found to be significantly increased in disease when

compared to health. These three species were grouped into the 'red complex' bacteria.

Chairside Diagnostic tools were available based on assays that detect these species. Red complex bacteria were thought to be the primary etiological agents for more than 30 years and therapeutic intervention to eradicate these species were also considered. The main advantage of this technique is that they are capable detecting multiple species from various samples in a same time.

Polymerase chain reaction:

Polymerase chain reaction (PCR) was first founded by **Mullis et al.**[97]. A part of gene or a specific gene is amplified which can be used to identify the bacterial species. PCR-based methods were used by researchers in their studies to detect specific species directly from oral samples. They focused mainly on the identification of a few species associated with the putative periodontal pathogens which includes Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola, and Aggregatibacter actinomycetemcomitans[28,75,101,116,138.]

In previous sequence analysis of 16S rRNA genes from oral microflora, a number of bacterial species were identified as candidates as putative pathogens for periodontitis, that includes the traditional species, such as P. gingivalis, T. denticola and T. forsythia[105].**Kumar PS**[68] stated that in

order to detect the prevalence of causative pathogens, healthy subjects and diseased plaque samples can be used with the help of species specific PCR primers.

Real -time polymerase chain reaction:

The technique is similar to PCR but it is capable of detecting the amplified DNA and helps in simultaneous quantification as the progress of reaction occurs in real-time[46,123].

Open-ended approaches:

This technique is advantageous now since it can detect previously unknown microbes including those which are non-cultivatable. These approaches are based on 16 S rRNA sequencing. This approach has been used to study the microbial population in different ecosystems, enabling the characterization of hitherto uncultivated microbial communities[41,102,121]. Using this approach, the diversity of different colonization niches in the oral cavity has been explored[13,58,108].

16S rRNA sequencing:

This technique is proven to be the most important phylogenetic marker that amplifies and analyse 16S rRNA genes in a plaque sample and it is a culture independent technique[135]. 16S rRNA gene sequence of bacterial

species can be recognized by PCR primers. Various microbes can be compared at different taxonomic levels with the help of 16S rRNA gene which consist about 1500 bases[72,109,124].

Next Generation Sequencing:

The next-generation sequencing works on the principle that it may involve oligonucleotide which undergoes cyclical ligation which is of machine automated or there will be a repeated cycles of polymerase-mediated nucleotide extensions[86, 128,150]. In a single machine run, there will be a huge amount of nucleotide sequence as millions of reactions occur in a massively parallel process. The types of NGS include:

1. Roche/454 FLX (Life Sciences, Branford, CT, **Margulies et al.**[87])
2. Illumina/ Solexa Genome Analyzer (Illumina, San Diego, CA, **Bentley DR**[14] , **Korbel et al.**[65])
3. SOLiD (Life Technologies, Carlsbad, CA, **Mardis**[86], **Voelkerding et al.**[150]).
4. 4)HiSeq and the Ion Torrent Personal Genome Machine (PGM) (**Rothberg et al.**[112]).

NGS consists of 2 themes which includes a bead to which the fragments are immobilized to the solid surface and DNA fragments are ligated oligonucleotide adaptors which serve as primers for sequencing and amplification.

The pyrosequencing technology is an additional theme in Roche / 454 which consists of beads which are available on picotiterplate wells which has a clonal amplification of templates, the laminar fluidics that control the delivery of deoxyribonucleotide triphosphate and the luminescent which burst upon deoxyribonucleotide triphosphate incorporation is detected bya high resolution charge-coupled device camera. Advantage of 454 sequencing is its long read lengths (400–500 nucleotides) and the amount of sequence generated (0.5 Gb)[84]. The long reads can handle repetitive regions better than other next generation sequencing systems[100].

SOLiD 35 nucleotide reads which is two-base encoding system and it provides better sequence fidelity than the one-base next-generation sequencing systems[150] but biased sequence coverage is obtained in AT-rich repetitive sequences[51].This technique requires long running time and only 35% of the raw reads are useable where as 95% of the raw reads can be used for the 454 system.

Illumina/Solexa Genome Analyzer

This technique is an highly targeted NGS approach which provides more sequence reads per run, than previous methods thereby allowing for more in depth coverage[14,14,64]. In this technique, there will be specific number of cycles, which detects fluorescent reversible-terminator nucleotides on clonally amplified DNA by a genome analyzer.

The target is first amplified in the Solexa system after which only one of the strands is sequenced with all four deoxyribonucleotide triphosphates in which each deoxyribonucleotide triphosphate has a unique fluorophore .In 454 system tis sequencing does not occur in one step. In order to prevent insertion of multiple nucleotide bases, reversible terminator nucleotides are present [91]and adaptor sequences are present at each end of the fragments which is immobilized with oligonucleotides that are grafted to the surface of a microfluidic chamber. The DNA templates are hybridized to the immobilized oligonucleotides by the adaptors after which they are copied using bridge amplification[3].

Fluorescently labelled deoxyribonucleotide triphosphates and the primer hybridize to one of the adaptors when there is an addition of polymerase there by initiating DNA sequencing. This is recorded bya charge-coupled device camera when there is a burst of light incorporation of a complementary base and the fluorophore is washed away unlike 454 sequencing and is removed from the incorporated base, washed away and the cycle is repeated[63,114].

The Solexa system has an advantage that 1.5 Gb of sequence per run can be achieved with read lengths which range from 35 to 100 bases out of which each run requires 3–5 days to complete[113].Whereas this system produce biased sequence coverage that occurs in AT-rich repetitive sequences as they have short read length[51,65].

This study used NGS to characterize the composition of the microbial communities of plaque samples from periodontal health and gingivitis.

Initially, the study of bacterial communities in the oral cavity was conducted with culture-dependent methods[96] . However, because 40% to 60% of bacteria in the oral cavity cannot be cultivated and so culture-independent methods such as denaturing gradient gel electrophoresis were used[42].

Although several metagenomic studies using 16S ribosomal RNA (rRNA) gene cloning techniques provided information about bacterial communities, the studies were limited to insufficient clone numbers and a lack of statistical significance[69]. To solve this problem, next-generation sequencing (NGS) has revolutionized bacterial diversity studies on the oral microbiota of subjects with a healthy or periodontitis status[2,45].

A number of previous high-throughput 16S rRNA sequencing studies characterized oral bacterial communities to the phylum or genus level only[60,71,76]. It is important to distinguish taxa at the species-level, as different species within the same phylum and/or genus may be health-associated or pathogenic/disease-associated. The targeting of a highly variable region of the 16S rRNA gene (V1–V3) and the use of a curated human oral 16S rRNA gene reference set (HOMD), enabled the identification of OTUs (clustered at a distance of 0.015) to species-level where possible.

Whilst some studies have also recently reported species-level 16S rRNA gene pyrosequencing analysis of the bacterial communities in periodontal health, gingivitis and chronic periodontitis, these studies were cross-sectional in nature and did not examine changes in the same individuals during the transition from health to disease[2,45,55]. In the present study a highly species-rich bacterial community (201–383 OTUs per sample) was revealed in early health-associated plaque.

The Human Oral Microbiome Database

The goal of creating the Human Oral Microbiome Database (HOMD) is to provide the scientific community with comprehensive information on the approximately 700 prokaryote species that are present in the human oral cavity. Approximately 54% are officially named, 14% unnamed (but cultivated) and 32% are known o-nly as uncultivated phylotypes. The HOMD presents a provisional naming scheme for the currently unnamed species so that strain, clone, and probe data from any laboratory can be directly linked to a stably named reference scheme[128,160].

The HOMD links sequence data with phenotypic, phylogenetic, clinical, and bibliographic information. Genome sequences for oral bacteria determined as part of this project, the Human Microbiome Project, and other sequencing projects are being added to the HOMD as they become available. Genomes for 400 oral taxa (58% of taxa on HOMD) are currently available on

HOMD. The HOMD site offers easy to use tools for viewing all publically available oral bacterial genomes.

Microbiome Studies In Periodontal Disease:

Chronic periodontitis:

Kroes et al. [66] conducted a study using 16S rRNA gene sequencing and Sanger sequencing in order to evaluate the subgingival microbiome in chronic periodontitis and founded that there were 77 phylotypes involved in the disease out of which 48% were novel.

Paster et al.[107] did a study using Sanger technique under different periodontal conditions and founded that there was a total of 347 phylotypes involved in the disease and among them 215 were novel.

Kumar et al.[67] founded that 274 phylotypes were associated in the chronic periodontitis while evaluating the subgingival microbiome using Sanger sequencing in health and periodontitis. Phylotypes associated with periodontitis were identified as Peptostreptococcus spp., Filifactoralocis, Megasphaera sp., Desulfobulbus sp., Dialister spp. Campylobacter spp., Selenomonas sp., Deferribacteres sp., Catonella sp., Tannerella forsythia, Streptococcus spp., Atopobium sp., Eubacterium sp. and Treponema sp. Phylotypes associated with health were Veillonella sp., Campylobacter gracilis, Campylobacter showae, Abiotrophiaadiacens,

Eubacteriumsaburreum, Gemella sp., Streptococcus sanguis, Streptococcus mutans, Capnocytophagagingivalis, Rothiadentocariosa, Eubacterium sp. and Selenomonas sp.

Griffen et al.[45]founded 16 phyla, 106 genera and 596 species in chronic periodontitis using high throughput sequencing. He also stated that health associated species may also be present in the disease but in low abundance.

Abusleme et al.[2] did a study in 22 subjects to understand the ecology of oral subgingival communities in health and periodontitis and elucidated the relationship between inflammation and the subgingival microbiome using 454-pyrosequencing of 16S rRNA gene libraries and quantitative PCR. He founded that periodontitis communities had higher proportions of Spirochetes, Synergistetes, Firmicutes and Chloroflexi, among other taxa, while the proportions of Actinobacteria, particularly Actinomyces, were higher in health. Total Actinomyces load, however, remained constant from health to periodontitis.

Hong et al.[52] founded that two variant of microbiome profiles can be seen in periodontitis which includes clusters A and cluster B which was derived clustering analyses of microbial abundance profiles. In subjects with cluster A communities there was an increased proportions of different periodontitis-associated species, health-associated species and core taxa which is prevalent both in health and periodontitis. Cluster B communities showed

increased proportions of certain periodontitis-associated organisms, such as Porphyromonas gingivalis, Tannerella forsythia and Treponema denticola, and taxa recently linked to periodontitis. The cluster B community showed a positive correlation with periodontitis extent.

Chi-Ying Tsai[146] did a study in Taiwanese individuals with chronic periodontitis with 16S rRNA metagenomic approach. He determined the subgingival microbiota and demonstrated a microbial shift from health to disease.

Camelo-Castillo[20] evaluated the relationship between the chronic periodontitis-associated subgingival microbiota and clinical inflammation. Subgingival bacterial samples from periodontal patients were studied by pyrosequencing PCR products of the 16S rRNA gene and by real-time PCR. He founded that 16S pyrosequencing revealed that increased inflammation, at sites with periodontitis, is associated with a more diverse subgingival microbiota and specific changes in the bacterial composition, involving "established" periopathogens, symbionts and novel low-abundance pathobionts.

Gingivitis:

Kistler et al.[61] in 40 patients studied using 454pyrosequencing of the 16S rRNA genes and bacterial culture, to characterize the composition of plaque during the transition from periodontal health to gingivitis and founded

that species-level phylotypes positively and negatively correlated with gingivitis. Increased community diversity and significant shifts in microbiome structure after two weeks of oral hygiene abstention was reported. All of the healthy volunteers developed gingivitis after two weeks. Pyrosequencing yielded a final total of 344 267 sequences after filtering, with a mean length of 354 bases, that were clustered into an average of 299 species-level Operational Taxonomic Units (OTUs) per sample. Changes in the relative abundance of OTUs during the transition from health to gingivitis were correlated to bleeding on probing (BoP) scores and resulted in the identification of new health and gingivitis associated taxa. Comparison of the healthy volunteers to the periodontitis patients also confirmed the association of a number of putative periodontal pathogens with chronic periodontitis. Taxa associated with gingivitis included Fusobacterium nucleatum subsp. polymorphum, Lachnospiraceae sp., Lautropia sp. and Prevotellaoulorum, whilst Rothiadentocariosa was associated with periodontal health.

Huang et al. [53] founded that 15 genera could distinguish healthy and gingivitis samples with 74 percent accuracy. Temporal shifts in community structure were observed along the progression from naturally occurring gingivitis to healthy gingiva to experimental gingivitis.

Park et al[104] analyzed bacterial communities in the subgingival paper point samples from 32 Korean individuals with no sign of disease, gingivitis, or periodontitis using 454 FLX Titanium pyrosequencing. A total of 256,113

reads representing 26 phyla, 433 genera, and 1,016 species were detected. Bacteroidetes, Fusobacteria, Synergistetes, and Spirochaetes were the abundant phyla in periodontitis subjects, whereas Firmicutes and Proteobacteria were identified as the dominant phyla in the gingivitis and healthy subjects, respectively. Although high levels of Porphyromonas, Fusobacterium, Fretibacterium, Rothia, Filifactor, and Treponema genera were observed in the periodontitis subjects, Streptococcus, Capnocytophaga, Leptotrichia, and Haemophilus genera were found at high frequency in the gingivitis subjects. Species including Porphyromonas gingivalis, Fusobacterium nucleatum, and Fretibacterium fastidiosum were significantly increased in periodontitis subjects. On the other hand, Streptococcus pseudopneumoniae, Haemophilus parainfluenzae, and Leptotrichiahongkongensis were preferentially observed in the gingivitis subjects. He founded that showed distinct microbial communities were present in health, gingivitis and periodontitis.

Huang et al[54] compared plaque microbiota changes in gingivitis patients by using tooth brush only in one group and dental scaling in another group among 91 subjects. He founded that only Actinobaculum, TM7 and Leptotrichia were consistently reduced by both the treatments, whereas the different microbial signatures of the two treatments during gingivitis relieve indicate distinct mechanisms of action. Therefore this study suggests that microbiota based signatures can serve as a valuable approach for

understanding and potentially comparing the modes of action for clinical treatments and oral-care products in the future.

Liam Shaw et al[120] did a study in 962 Malawian women for periodontal disease and used 16S rRNA gene amplicon sequencing to estimate the bacterial compositions of supragingival plaque samples.Associations between bacterial relative abundances and gingivitis /periodontitis were investigated. He founded that the main differences in supra-gingival plaque compositions were associated more with gingivitis than periodontitis, including higher bacterial diversity and a greater abundance of particular species. However, even after controlling for gingivitis, the presence of subgingival periodontitis was associated with an altered supra gingival plaque. A small number of species were associated with periodontitis but not gingivitis, including members of Prevotella, Treponema and Selenomonas, supporting a more complex disease model than a linear progression following gingivitis. This finding confirmed that periodontitis cannot be considered simply an advanced stage of gingivitis even when only considering supra gingival plaque.

Diaz et al[34] conducted a study using high-throughput 16S rRNA gene sequencing in order to evaluate subgingival microbiome shifts from health to periodontitis. He founded that the development of gingivitis is also characterized by distinct shifts. Microbiome shifts resemble microbial successions and result from interspecies interactions and community

adaptation to the changing environment as inflammation ensues. Gingivitis-associated and core species are proposed as likely mediators of microbiome transitions.

Andre Paes Batista da Silva et al[7] did a study in 175 subjects which measured microbial composition changes during biofilm overgrowth and subsequent removal among patients with various states of periodontal disease using 16S rRNA probes (human oral microbe identification microarray) who were abstained from oral hygiene while using an acrylic stent. At day 21, participants reinstituted oral hygiene and were followed for 4 weeks.

He founded that Synergistetes is associated with more severe forms of periodontal disease and plaque accumulation. When oral hygiene was resumed and another examination performed after 4 weeks, most bacteria in all groups were reduced to base line levels. However, some bacterial phyla did not reduce to baseline levels, such as synergistetes. Most importantly, higher levels of fusobacteria, proteobacteria, firmicutes, synergistetes, and bacteroidetes are associated with incident increasing PD. He concluded that these findings further support the potential critical role of synergistetes as well as the previously characterized fusobacteria, proteobacteria, firmicutes, and bacteroidetes in periodontal pathogenesis.

Ke Deng et al.[29] with gingivitis analysed microbiome in 54 patients with gingivitis from subgingival plaque samples among Chinese population using high throughput 16s rRNA sequencing extracting 35 genera and 46 species. He proposed that there was a significantly different microbial community composition of sub gingival plaque between health and gingivitis. He also showed that the presence of P. gingivalis present among the person aged 18 to 21 had a high risk of developing periodontitis.

Materials and Methods

MATERIALS AND METHODS

Study population

A sample size of 8 patients was chosen for the study due to the cost and complexity of the technique involved, which is in accordance with previous studies by **Zheng et al[161], Dzink et al.**[39]

Eight individuals, who reported to the outpatient Department of Periodontics in Ragas Dental College and Hospital Chennai, were enrolled in the present study, of which 4 were periodontally healthy individuals (control group) and 4 were gingivitis patients (test group).

Control group consisted of subjects with clinically non-inflamed, healthy gingiva that had negligible bleeding on probing <10%, probing pocket depth {PPD} ≤3mm and no clinical attachment loss {CAL}.

Test group consisted of subjects with gingivitis who had bleeding on probing {BOP}> 20%, with PPD≤ 3mm and no clinical attachment loss.

The study protocol was explained, and written informed consent was received from each individual before clinical periodontal examination and plaque sampling along which the medical and dental histories were obtained.

Inclusion criteria

- Subjects must not have any contributing systemic illness
- Subjects must meet the criteria of periodontal health and gingivitis as described above was included in this study.

Exclusion criteria

- Patient with systemic disorders, such as diabetes mellitus, immunological disorders or HIV that may be a contributing factor for periodontal disease.
- Patient who were under medication drugs that alter the microbial characteristics which includes immunosuppressant drugs or steroids.
- Smokers or tobacco users.
- Patient with previous history of periodontal treatment in the past 6 months.
- Patient who had been prescribed antimicrobial therapy for past 6 months.

Subgingival plaque sampling

All examinations were performed by a single, calibrated examiner. For the diseased samples, the sites that exhibited bleeding on probing were selected and the sample was collected in a single Eppendorf tube. The site to be sampled was first isolated using cotton rolls and air dried gently. Supragingival plaque was gently removed with a gauze and subgingival

plaque was obtained from the sample teeth and with sterilized Gracey curettes. The plaque from within 2 mm of the sulcus depth was carefully obtained with a gentle pull motion towards the coronal surface. The tip of the curette was then inserted in the Eppendorf tube containing ionized molecular water and shaken until the plaque was removed from the curette. For the healthy plaque samples, sites that did not exhibit any sign of inflammation and bleeding on probing were chosen. The same procedures were followed for sampling from these sites.

The samples obtained were frozen and stored at -20°C until the sample collection period was completed. All the samples were collected within 2 days and then sent for processing so as to avoid any degradation.

DNA extraction, 16S rRNA amplification, library construction and sequencing

Genomic DNA was extracted from 8 plaque samples of gingivitis and periodontally healthy patients with the Fast DNA kit and the FastPrep24-5G instrument according to manufacturer's recommendations (MP Biomedicals, Santa Ana, CA).

Extracted DNA was purified with silica-based spin filters (FastDNA kit) and DNA was amplified using the 16S V3 (341F) forward and V4 (805R) reverse primer pairs with added Illumina adapter overhang nucleotide sequences.

Amplicon synthesis was performed using thermocycling with 8.5 µl of genomic DNA, 2 µl of amplicon PCR forward primer (2.5 µM), 2 µl of amplicon PCR reverse primer (2.5 µM), and12.5 µl of 2x KAPA HiFiHotStart Ready Mix (KapaBiosystems) at 95 °C initial denaturation for 3 min, followed by 25 cycles of 95 °C for 30 s, 62.3 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

Reactions were cleaned up with AgencourtAMPure XP beads (Beckman Coulter Genomics) according to the manufacturer's protocol. Attachment of dual indices and Illumina sequencing adapters was performed using 5 µl of amplicon PCR product DNA, 5 µl of IlluminaNextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFiHotStart Ready Mix, and 10 µl of PCR-grade water (UltraCleanDNA-free PCR water; MO BIO Laboratories, Inc., Carlsbad, CA, USA), with thermocycling at 95 °C for 3 min, followed by 8 cycles of 95 °C for 30s, 55 °C for 30s, and 72 °C for 30s, and a final extension at 72 °C for 5 min.

Constructed 16S metagenomic libraries were purified with AgencourtAMPure XP beads and quantified with Quant-iTPicoGreen and the KAPA Library Quantification Kit (KAPABIOSYSTEMS). Library quality control was performed with the Agilent Technologies 2100 Bioanalyzer to ascertain quality and average size distribution.

Samples were denatured and diluted to a final concentration of 10 pM with a 20 % PhiX (Illumina) control. Sequencing was performed using the IlluminaNextseq 500 System. All 8 samples were multiplexed and sequenced in a single lane on the NextSeq using 2 × 150 bp paired-end sequencing.Data analysis was done by using 16s metagenomics tool from Base Space Onsite Operational taxonomic units (OTUs) were assigned to each sequence using HOMD database.

Statistical analysis

Conventional statistical analysis cannot be done in this study due to individual variation in the microbial community present in the sub gingival samples and these data was evaluated according to the previous researchers [45,68,78]. Comparison of abundance among the various species was done by Mann-Whitney U test and Wilcoxon W test.

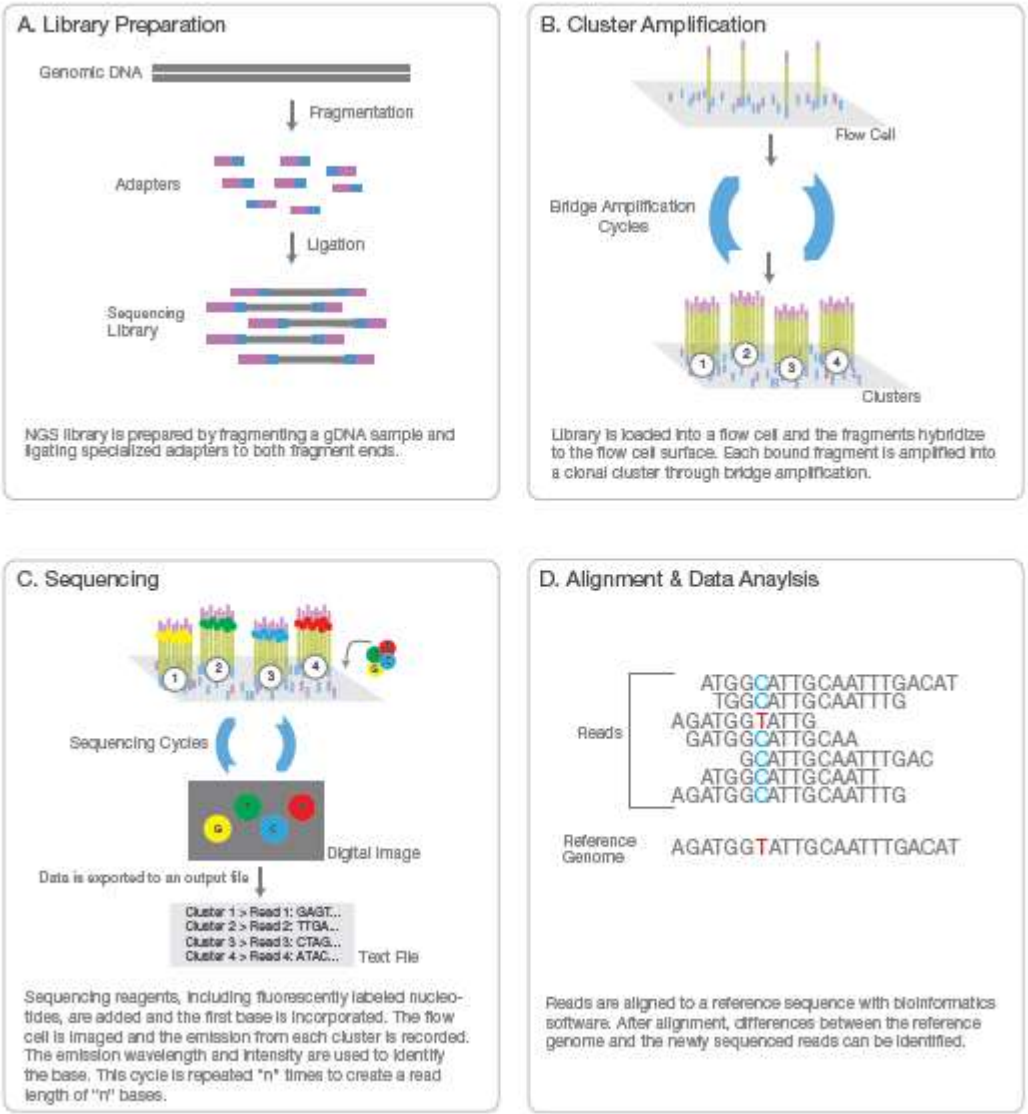
At the genus level, circular maximum likelihood phylogenetic tree was constructed using iTOL and PhyloT tools as per Letunic and Bork[73].

Photographs

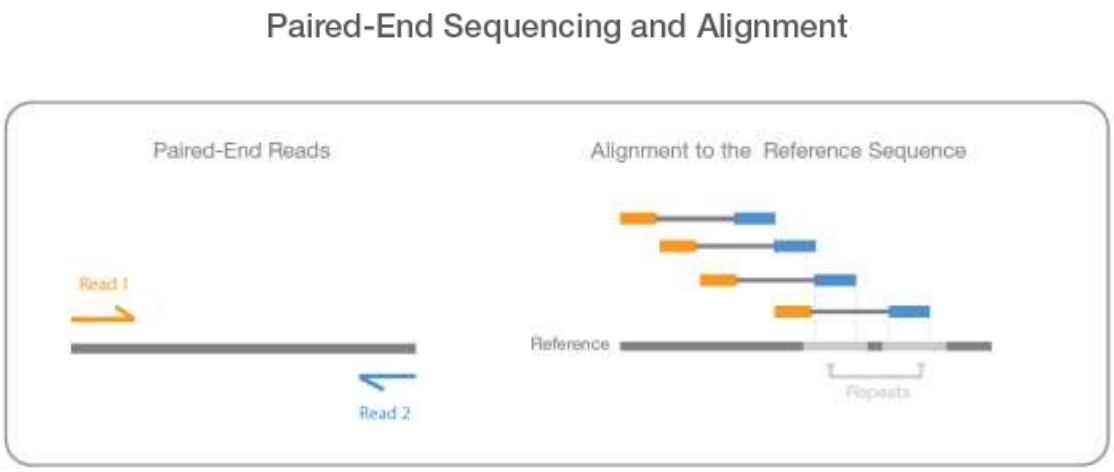
SAMPLE COLLECTION


FastStart Essential DNA
06 924 492 001

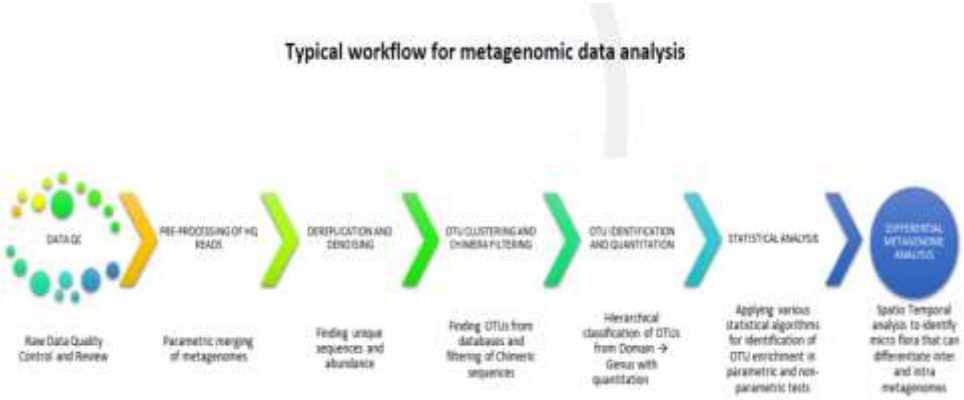
ILLUMINA SEQUENCING



4 basic steps in Illumina NGS work flow


Paired-End Sequencing and Alignment
Paired-End Reads
Alignment to the Reference Sequence
Read 1
Read 2
Reference
Repeats


NGS Library Preparation Kits
Illumina Sequencing Instruments
Automated Data Analysis Tools
5–6 Hours
1–2 Days
1–2 Days


Typical workflow for metagenomic data analysis
DATA QC
PRE-PROCESSING OF HQ READS
DEREPLICATION AND DENOISING
OTU CLUSTERING AND CHIMERA FILTERING
OTU IDENTIFICATION AND QUANTITATION
STATISTICAL ANALYSIS
Raw Data Quality Control and Review
Parametric merging of metagenomes
Finding unique sequences and abundance
Finding OTUs from databases and filtering of Chimeric sequences
Hierarchical classification of OTUs from Domain → Genus with quantitation
Applying various statistical algorithms for identification of OTU enrichment in parametric and non-parametric tests
Spatio Temporal analysis to identify micro flora that can differentiate inter and intra metagenomes

Results

RESULTS

The study population consisted of 4 healthy individuals and 4 gingivitis patients, whose subgingival plaque samples were obtained and subjected to 16s rRNA sequencing using NGS technology.

For each of the 8 plaque samples, bacterial phyla, genera and species were identified and their relative abundance quantified via taxonomic assignment against reference database

Healthy group:

There were totally 6 phyla, 21 genera and 37 species found in the healthy samples.

At the phylum level among the healthy samples Firmicutes were found more in abundance, followed by Proteobacteria and Candidatus Saccharibacteria which were about 30%, 23% and 18% respectively as shown in table 1-A and figure 1-A.

At genus level (top 8) among the healthy samples Veillonella was found more in abundance followed by Neisseria and Saccharibacteria genera incertae sedis which were about 22%, 15% 13% respectively as shown in table 2-A and figure 2-A.

At the species level(top 8) among the health samples Veillonella tobetsuensis had more abundance followed by Veillonella parvula and

Kingella oralis which included 21%. 14% and 11% respectively as shown in table 3-A and figure 3-A. Unclassified species accounted for about 16% among the top 8 species in health.

Gingivitis group:

There were a total of 7 phyla, 32 genera and 59 species found in the gingivitis samples.

At the phylum among the gingivitis samples Firmicutes was more in abundance of about 24% followed by Candidatus Saccharibacteri 20% and Proteobacteria 17% as shown in table 1-B and figure 1-B.

At the genus level(top 8) among the gingivitis samples Veillonella, Saccharibacteria genera incertae sedis and Dialister had more abundance of about 17% 16% and 14% respectively as shown in table 2-B and figure 2-B.

At the species level(top 8) among the gingivitis samples Veillonella tobetsuensis had more abundance of 17%, followed by Dialister invisus 15% and Anaeroglobus geminatus 14% as shown in table 3-B and figure 3-B. Unclassified species contributed for about 16% among the top 8 species in gingivitis.

Comparison of abundance of phyla among health and gingivitis:

The comparison of abundance of phyla between the health and disease group is represented in graph 1. There was more abundance of Firmicutes

and proteobacteria in the healthy group as compared to the disease group. The abundance of Candidatus Saccharibacteria, Actinobacteria, Bacteroidetes and Fusobacteria were found to be higher in the disease group as compared to the healthy group. Synergistetes was found only in the diseased group.

Comparison of abundance of top 20 species in health vs gingivitis:

The comparison of abundance of top 20 species present in health vs gingivitis has been compared as shown in table 4-A and graph 2-A. The mean standard deviation value for abundance of species in health is 0.925336 ± 0.7269395 and gingivitis is 0.865053 ± 0.6018219. The comparison of abundance of top 20 species in health vs gingivitis was not statistically significant at p=0.276 as shown in graph 2-B.

Comparison of abundance of top 20 species in gingivitis vs health:

Comparison of abundance of top 20 species present in gingivitis vs healthhas been compared in table 4-B and graph 3-A. The mean standard deviation value for abundance of species in disease is 0.847391 ± 0.5677049 and health is 0.726773 ± 0.7757812.The comparison of abundance of top 20 species in gingivitis vs health was not statistically significant at p=0.881 as shown in the graph 3-B

There are about 11 species present in the healthy group but absent in the gingivitis group that has been listed in the table 5-A. There are about 46 species that are present in the gingivitis group but are absent in the healthy

group as shown in table 5-B. These organisms may be the causative factor for the gingivitis.

Comparison of subgingival microbiome in healthversus gingivitis with circular maximum likelihood phylogenetic tree at genus level:

The subgingival microbiome was compared between healthy and gingivitis sites at genus level and is represented in the form of a circular phylogenetic tree in figure 3. The tree has been constructed with phyloT software and is displayed using iTOL as per **Letunic and Bork**[73]. The bars in the outer band (green) represent the relative abundance of bacterial genera in healthy (orange) and gingivitis (blue) sites.

Tables & Graphs

TABLES AND GRAPHS

Table 1-A: Evaluation of abundance of phyla and their percentage among the health samples

Sno	Phylum	Relative abundance expressed in logs	Percentage of phyla
1	Firmicutes	3.056	30%
2	Proteobacteria	2.392	23%
3	Candidatus Saccharibacteria	1.799	18%
4	Actinobacteria	1.633	16%
5	Bacteroidetes	1.342	13%
6	Fusobacteria	0.004	0%

Figure 1-A: Comparison of percentage of phyla in health

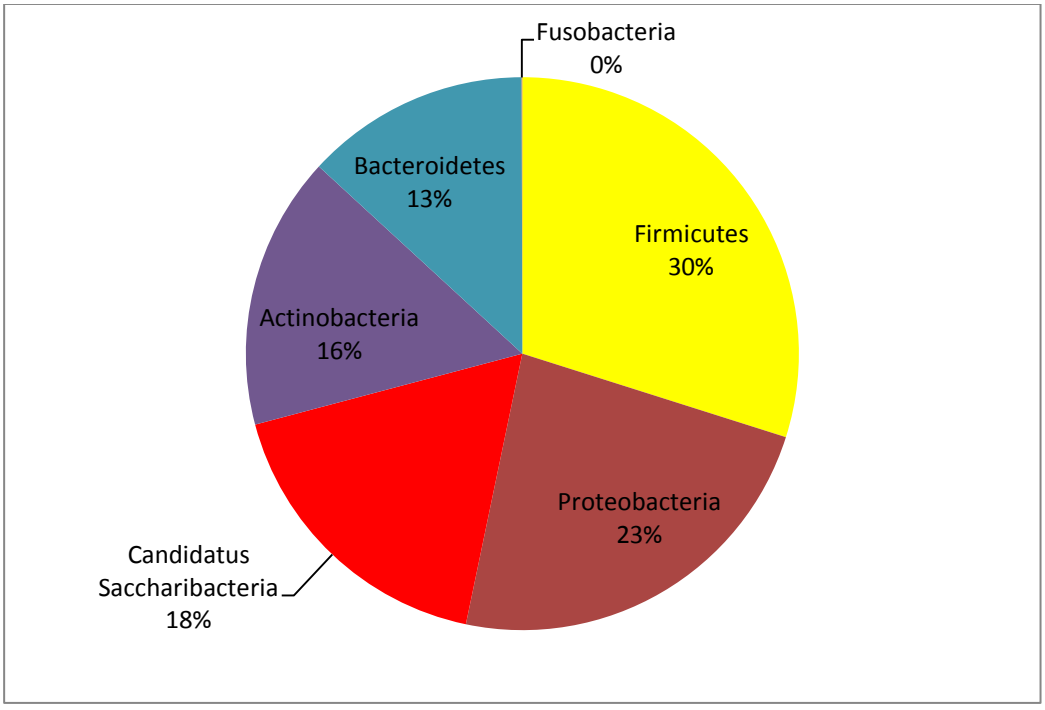


Table 1-B: Evaluation of abundance of Phyla and their percentage among gingivitis samples

Sno	Phylum	Relative abundance expressed in logs	Percentage of Phyla
1	Firmicutes	2.775	24%
2	Candidatus Saccharibacteria	2.303	20%
3	Proteobacteria	2.021	17%
4	Actinobacteria	1.653	14%
5	Bacteroidetes	1.447	13%
6	Synergistetes	1.255	11%
7	Fusobacteria	0.069	1%

Figure 1-B: Comparison of percentage of phyla in gingivitis

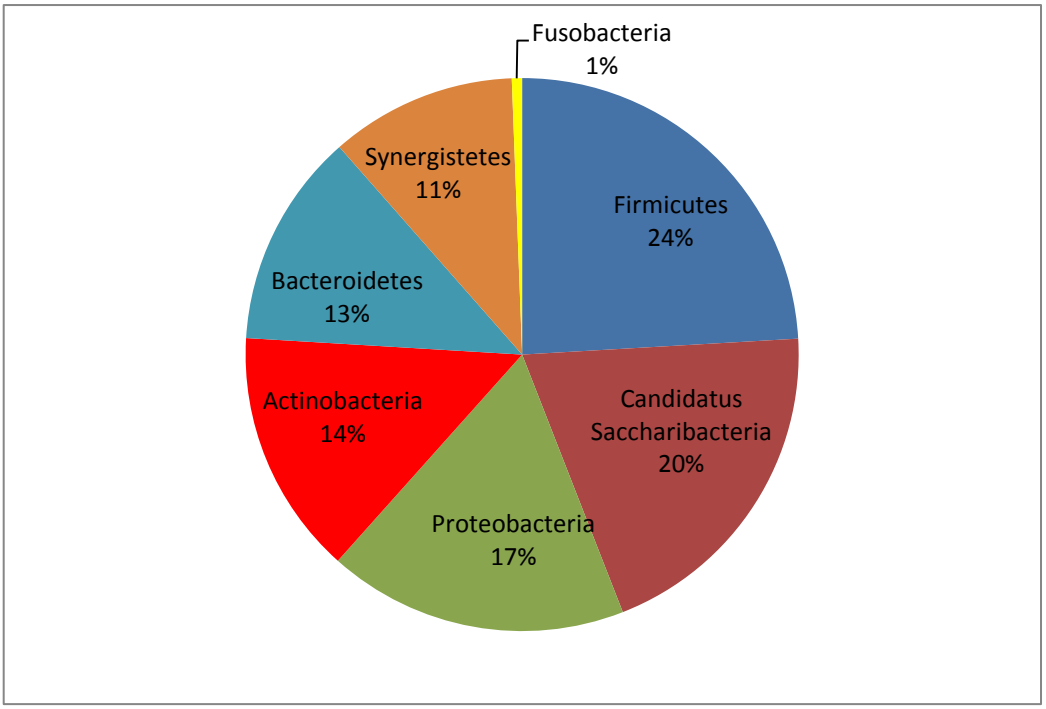


Table 2-A: Evaluation of abundance of top 8 Genera and their percentage among the health samples

Sno	Genus	Relative Abundance expressed in logs	Percentage of genera
1	Veillonella	3.050	22%
2	Neisseria	2.136	15%
3	Saccharibacteria genera incertae sedis	1.799	13%
4	Actinomyces	1.623	12%
5	Haemophilus	1.591	11%
6	Kingella	1.591	11%
7	Eikenella	1.255	9%
8	Prevotella	1	7%

Figure 2-A: Comparison of percentage of top 8 genera in health

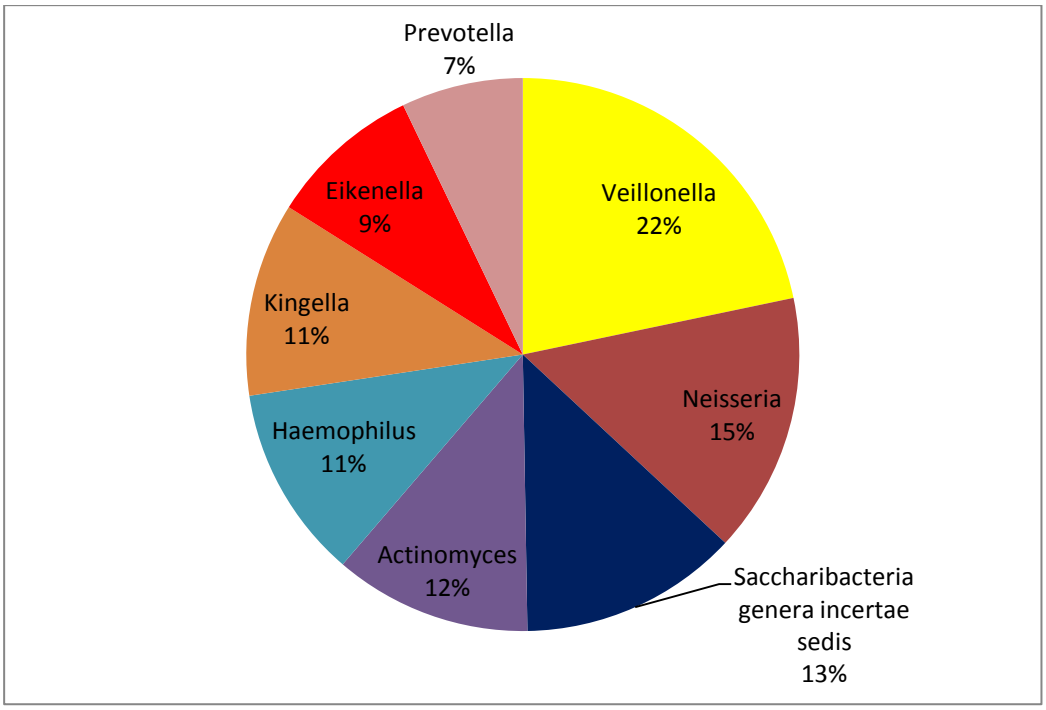


Table 2-B: Evaluation of abundance of top 8 Genera and their percentage among gingivitis samples

Sno	Genus	Relative abundance expressed in logs	Percentage of genera
1	Veillonella	2.514	17%
2	Saccharibacteria genera incertae sedis	2.303	16%
3	Dialister	2.086	14%
4	Anaeroglobus	1.995	14%
5	Kingella	1.462	10%
6	Eikenella	1.380	10%
7	Prevotella	1.380	10%
8	Neisseria	1.342	9%

Figure 2-B: Comparison of percentage of top 8 genera in gingivitis

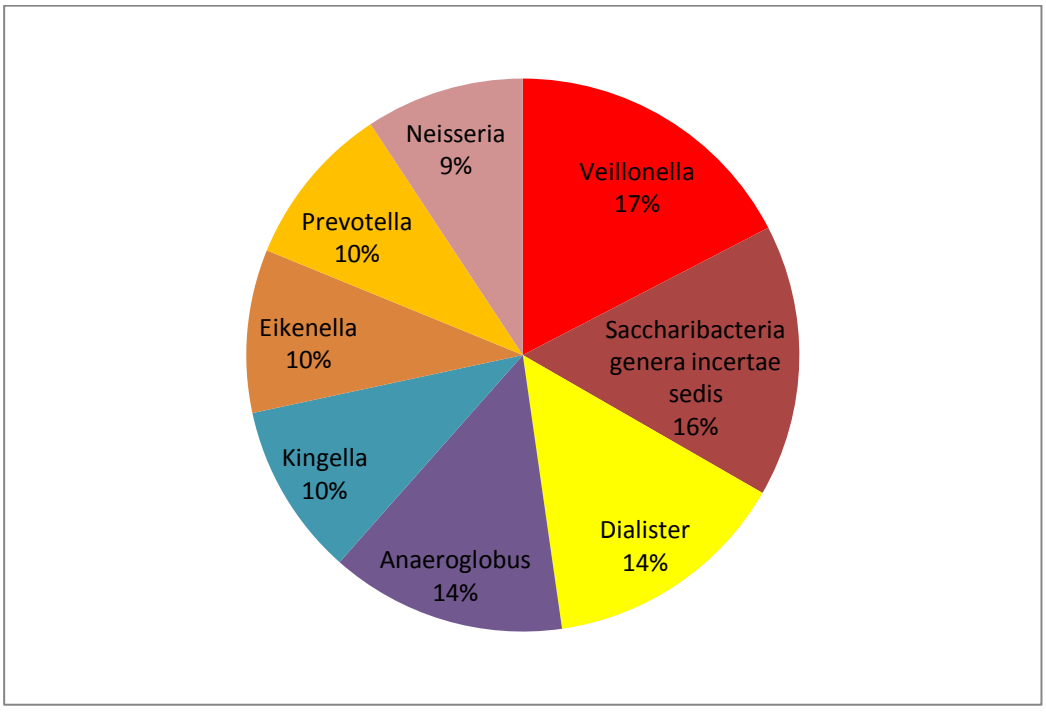


Table 3-A: Evaluation of abundance of top 8 Species and their percentage among health samples

Sno	Species	Relative abundance expressed in logs	Percentage of species
1	Veillonella tobetsuensis	3.003	21%
2	Unclassified	2.285	16%
3	Veillonella parvula	2	14%
4	Kingella oralis	1.591	11%
5	Haemophilus parainfluenzae	1.579	11%
6	Actinomyces odontolyticus	1.477	11%
7	Eikenella corrodens	1.255	9%
8	Actinomyces meyeri	0.954	7%

Figure 3-A: Comparison of percentage of top 8 species in health

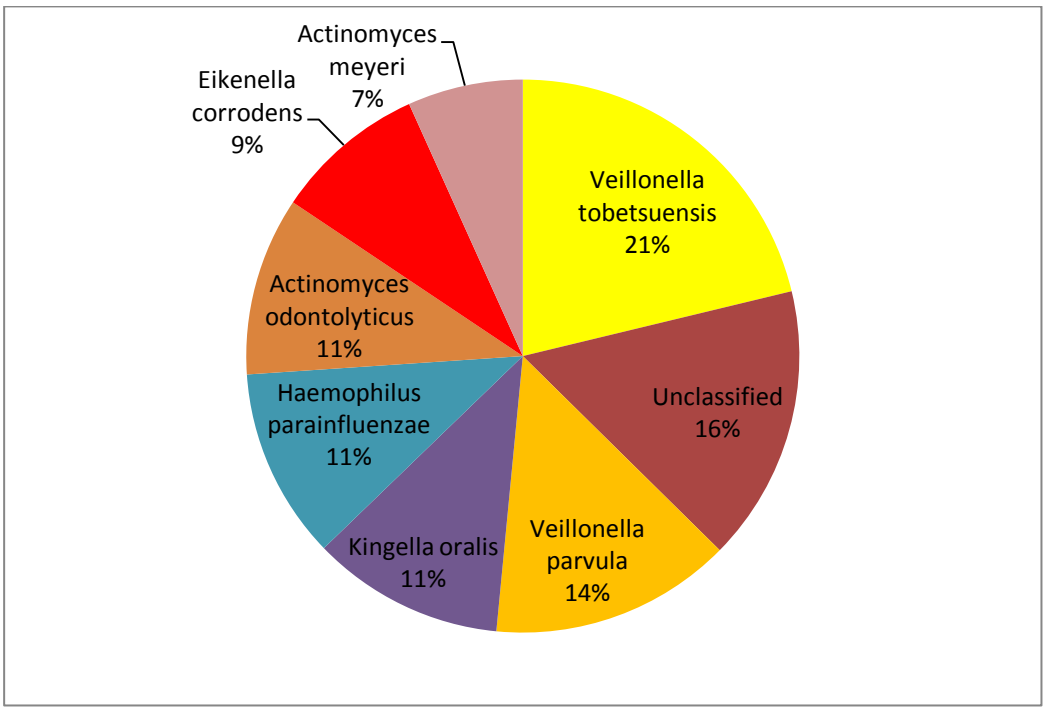
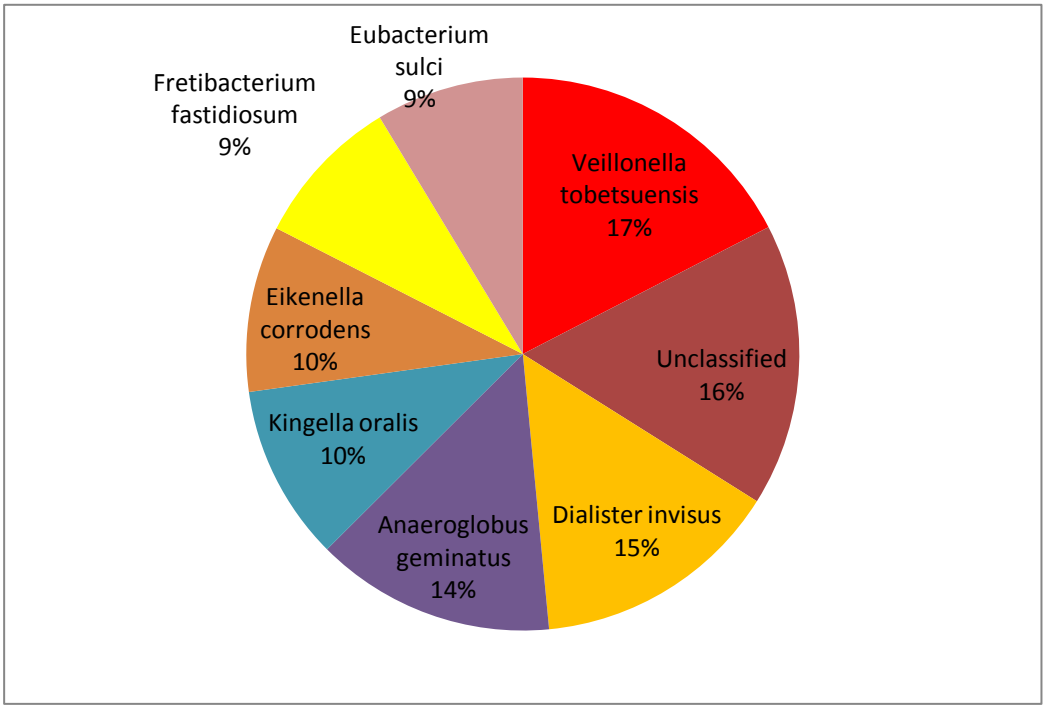


Table 3-B: Evaluation of abundance of top 8 Species and their percentage among gingivitis samples

Sno	Species	Relative abundance expressed in logs	Percentage of species
1	Veillonella tobetsuensis	2.475	17%
2	Unclassified	2.348	16%
3	Dialister invisus	2.068	15%
4	Anaeroglobus geminatus	1.995	14%
5	Kingella oralis	1.462	10%
6	Eikenella corrodens	1.380	10%
7	Fretibacterium fastidiosum	1.255	9%
8	Eubacterium sulci	1.230	9%

Figure 3-B: Comparison of percentage of top 8 species in gingivitis



Graph 1: Comparison of abundance of phylum in health and gingivitis

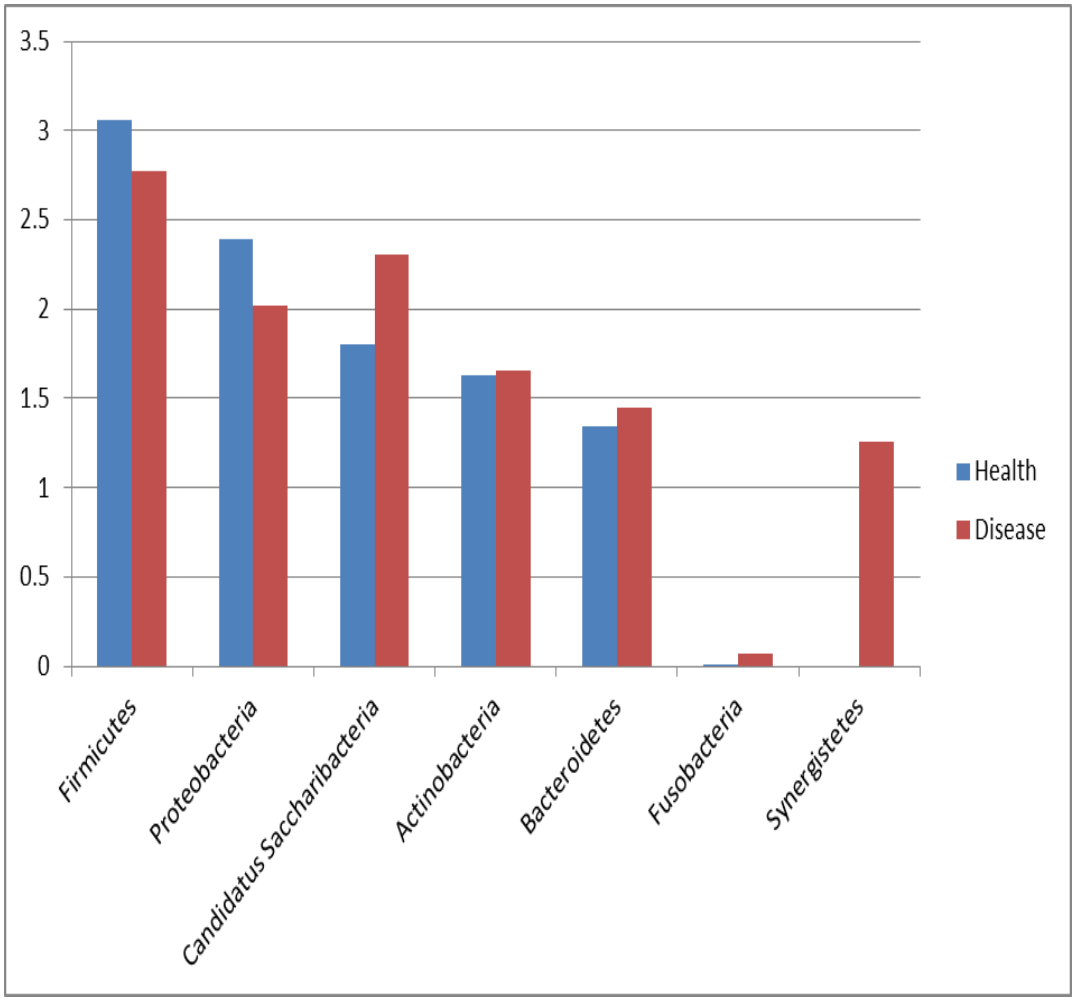
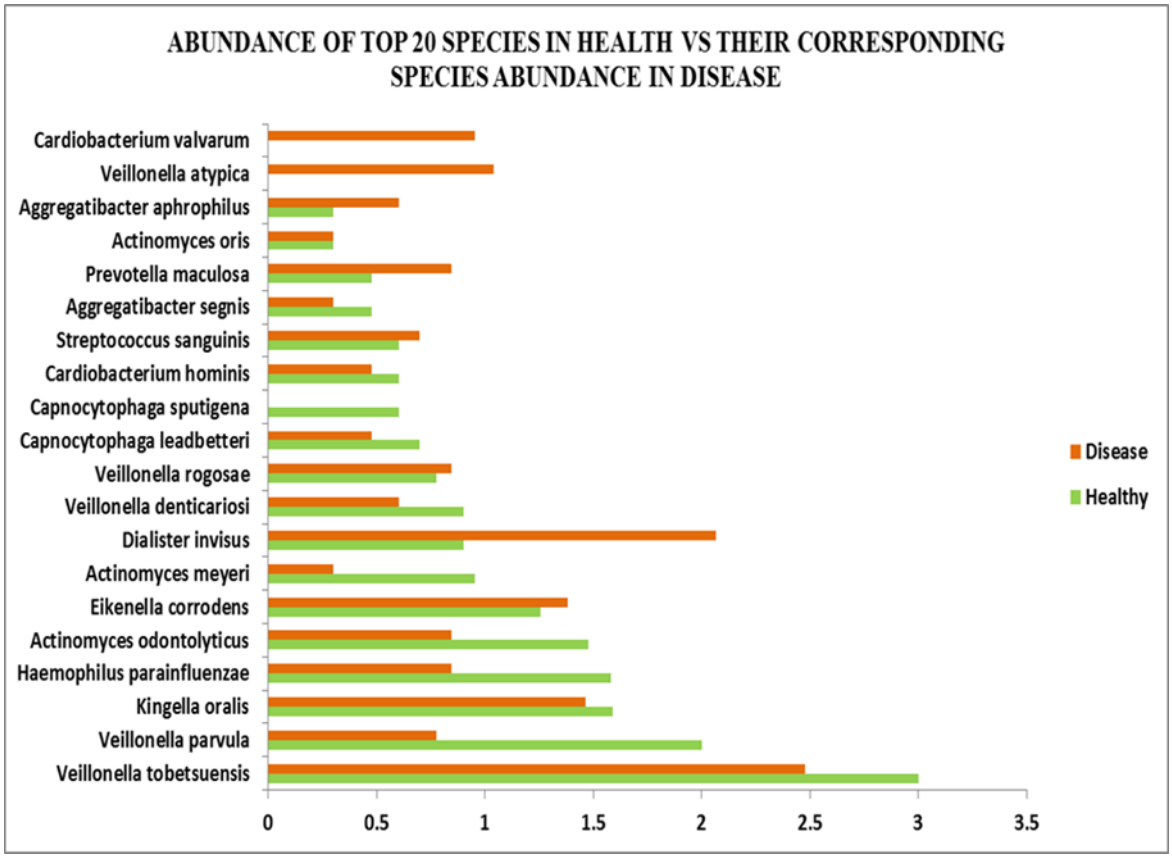


Table 4-A: Comparison of abundance of top 20 species in health vs gingivitis

Sno	Organism	Health abundance	Disease abundance
1	Veillonella tobetsuensis	3.003	2.475
2	Veillonella parvula	2	0.778
3	Kingella oralis	1.591	1.4623
4	Haemophilus parainfluenzae	1.579	0.845
5	Actinomyces odontolyticus	1.477	0.845
6	Eikenella corrodens	1.255	1.380
7	Actinomyces meyeri	0.954	0.301
8	Dialister invisus	0.903	2.068
9	Veillonella denticariosi	0.903	0.602
10	Veillonella rogosae	0.778	0.845
11	Capnocytophaga leadbetteri	0.698	0.477
12	Capnocytophaga sputigena	0.602	0.004
13	Cardiobacterium hominis	0.602	0.477
14	Streptococcus sanguinis	0.602	0.698
15	Aggregatibacter segnis	0.477	0.301
16	Prevotella maculosa	0.477	0.845
17	Actinomyces oris	0.301	0.301
18	Aggregatibacter aphrophilus	0.301	0.602
19	Veillonella atypica	0.004	1.041
20	Cardiobacterium valvarum	0.004	0.954

Graph 2-A: Comparison of abundance of top 20 species in health vs gingivitis



Graph 2-B: Total comparison of abundance of top 20 species in health vs gingivitis

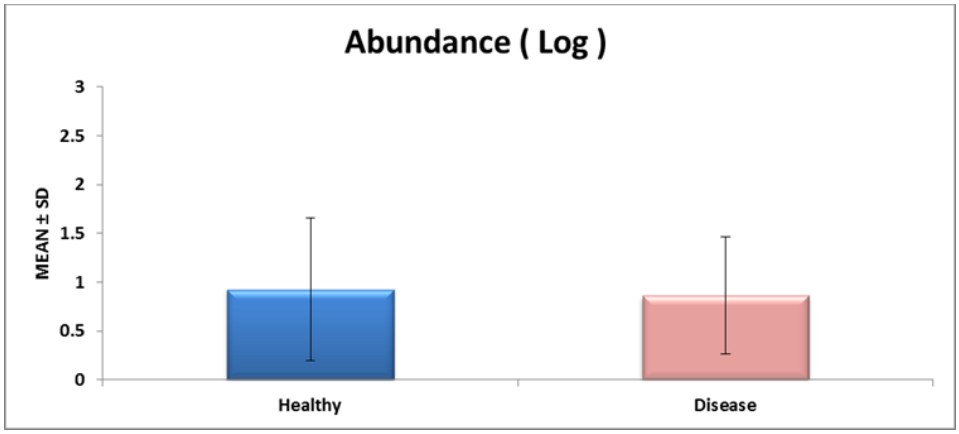
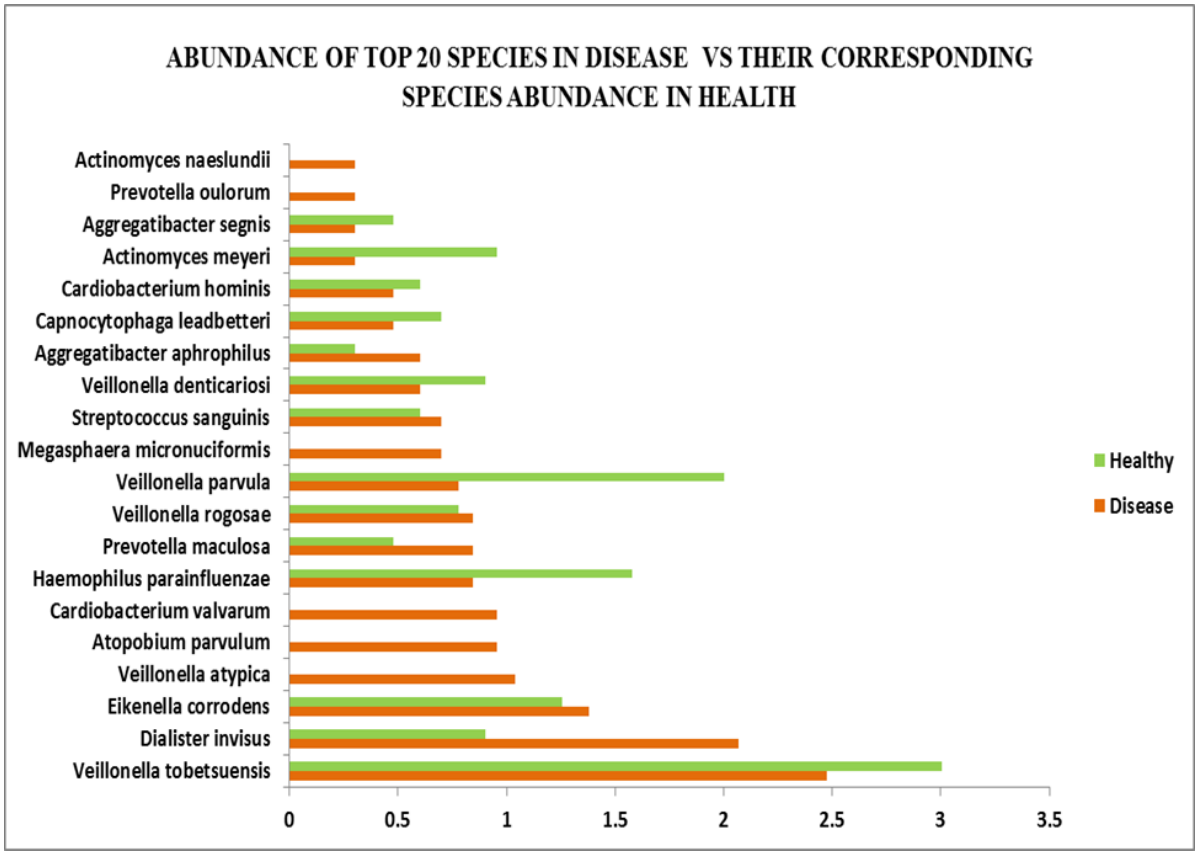


Table 4-B: Comparison of abundance of top 20 species in gingivitis vs health

Sno	Organism	Disease abundance	Health abundance
1	Veillonella tobetsuensis	2.475	3.003
2	Dialister invisus	2.068	0.903
3	Eikenella corrodens	1.380	1.255
4	Veillonella atypica	1.041	0.004
5	Atopobium parvulum	0.954	0.004
6	Cardiobacterium valvarum	0.954	0.004
7	Haemophilus parainfluenzae	0.845	1.579
8	Prevotella maculosa	0.845	0.477
9	Veillonella rogosae	0.845	0.778
10	Veillonella parvula	0.778	2
11	Megasphaera micronuciformis	0.698	0.004
12	Streptococcus sanguinis	0.698	0.602
13	Veillonella denticariosi	0.602	0.903
14	Aggregatibacter aphrophilus	0.602	0.301
15	Capnocytophaga leadbetteri	0.477	0.698
16	Cardiobacterium hominis	0.477	0.602
17	Actinomyces meyeri	0.301	0.954
18	Aggregatibacter segnis	0.301	0.477
19	Prevotella oulorum	0.301	0.004
20	Actinomyces naeslundii	0.301	0.004

Graph 3-A: Comparison of abundance of top 20 species in gingivitis vs health



Graph 3-B: Total comparison of abundance of top 20 species in gingivitis vs health

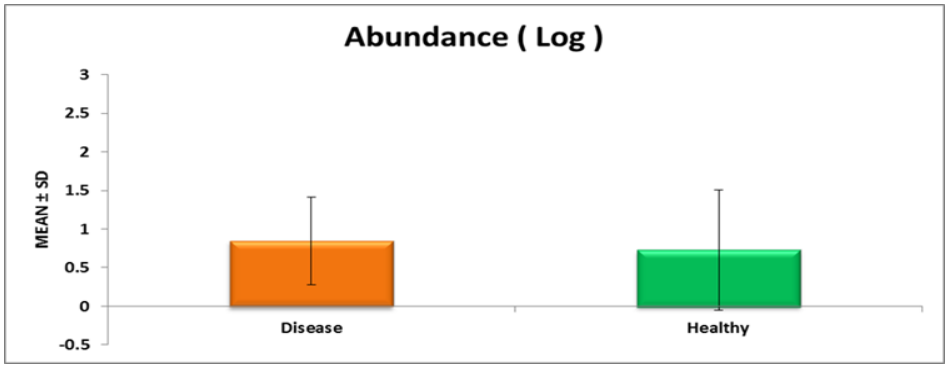


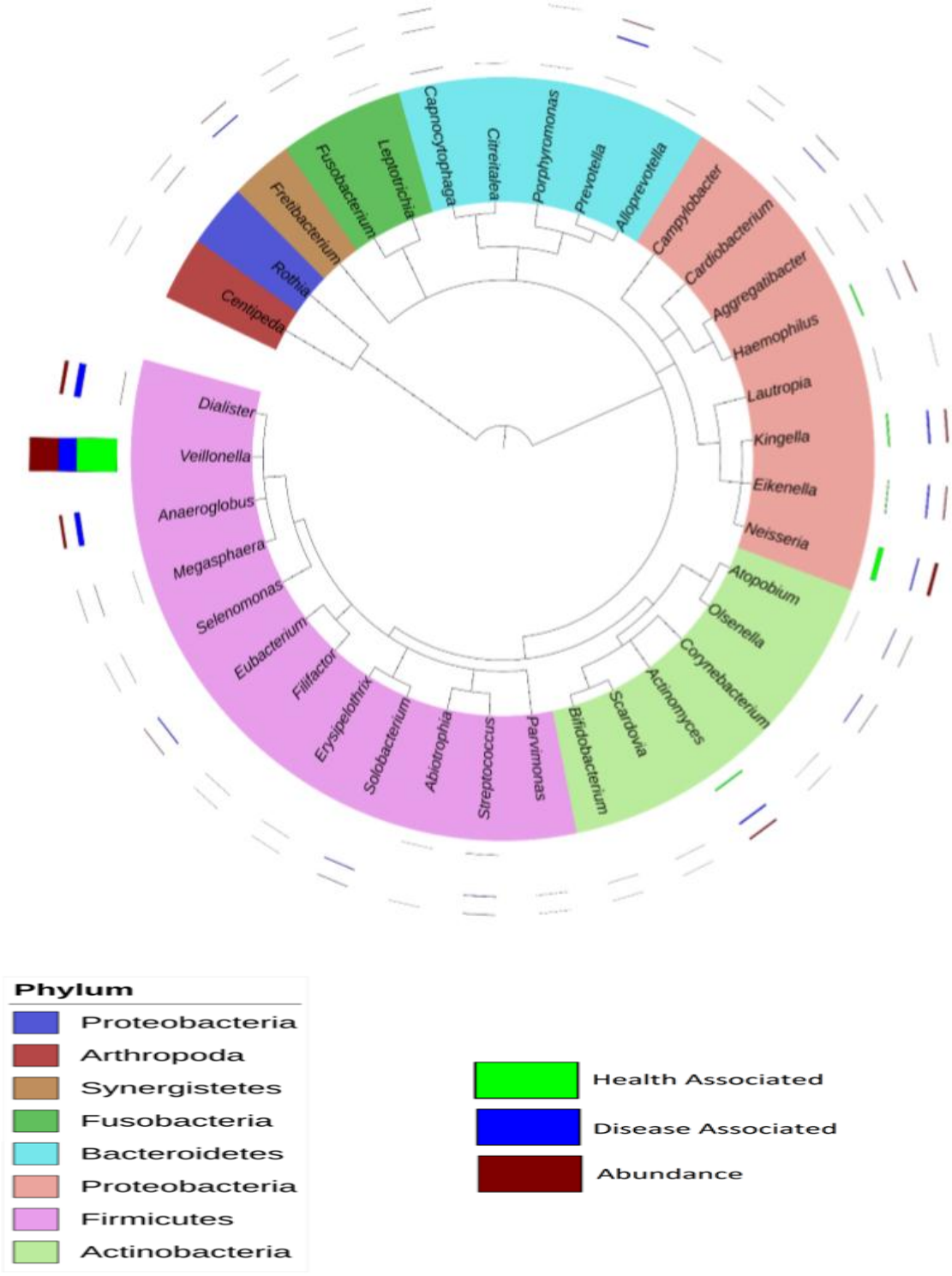
Table 5-A: Species present in health but not present in gingivitis

1	Abiotrophia defective
2	Alloprevotella rava
3	Citreitalea marina
4	Haemophilus pittmaniae
5	Lautropia mirabilis
6	Neisseria subflava
7	Porphyromonas catoniae
8	Prevotella loescheii
9	Prevotella nigrescens
10	Prevotella ruminicola
11	Streptococcus sinensis

Table 5-B: Species present in gingivitis but not present in health

1	Actinomyces cardiffensis	24	Dialister pneumosintes
2	Actinomyces gerencseriae	25	Dialister succinatiphilus
3	Actinomyces johnsonii	26	Eikenella corrodens
4	Actinomyces meyeri	27	Erysipelothrix rhusiopathiae
5	Actinomyces naeslundii	28	Eubacterium sulci
6	Actinomyces odontolyticus	29	Fretibacterium fastidiosum
7	Actinomyces oris	30	Fusobacterium periodonticum
8	Actinomyces viscosus	31	Fusobacterium simiae
9	Aggregatibacter aphrophilus	32	Haemophilus aegyptius
10	Aggregatibacter segnis	33	Neisseria oralis
11	Anaeroglobus geminatus	34	Neisseria polysaccharea
12	Atopobium parvulum	35	Olsenella uli
13	Atopobium rimae	36	Olsenella umbonata
14	Bifidobacterium dentium	37	Parvimonas micra
15	Campylobacter concisus	38	Prevotella enoecaPrevotella multiformis
16	Campylobacter showae	39	Prevotella oulorum
17	Campylobacter subantarcticus	40	Prevotella shahii
18	Capnocytophaga leadbetteri	41	Rothia aeria
19	Capnocytophaga sputigena	42	Scardovia wiggsiae
20	Cardiobacterium hominis	43	Selenomonas noxia
21	Cardiobacterium valvarum	44	Solobacterium moorei
22	Centipeda periodontii	45	Streptococcus mutans
23	Corynebacterium durum	46	Streptococcus pseudopneumoniae

Figure 4: Circular maximum likelihood of Phylogenetic tree at the genus level



Discussion

DISCUSSION

Periodontitis is a chronic inflammatory condition that affects the attachment apparatus of the teeth with the microbial communities in the plaque biofilm that inhabit the subgingival environment serving as the etiological agent. Though it is known to be an initiating or a triggering agent, the resultant disease is due to interplay of a myriad of factors viz. the inflammatory response, environmental, systemic and genetic factors of the host.

Gingivitis is a form of periodontal disease with reversible inflammation characterized by bleeding on probing and other clinical changes like colour and textural changes of gingiva[9]. In susceptible individuals, persistent or uncontrolled gingivitis leads or progresses to periodontitis.

The earliest studies proposed that the development of periodontitis was associated with an increase in the microbial load and later the subgingival microbial composition shifts from a population dominated by gram positive microorganisms to one with increased number of gram negative anaerobes[1,26]. However, these studies have been based on microbiologic study methods like culture, immuno diagnostic methods, DNA-DNA hybridization technique etc. The DNA-DNA checkerboard analysis have shown the presence, levels and properties of certain specific species associated with the classic clinical sign of periodontal disease and were termed as red complex organisms which includes

Porphyromonas gingivalis, Tanerella forsythia and Treponema denticola which was coined by **Sockransky**[130].

Though the initial studies confirmed the essential role of plaque biofilm using the experimental gingivitis model, the later studies provided more information regarding the specific bacterial species present and the fact that more than half of the bacteria present in the biofilm could not be cultivated in the laboratory[5,96]. Therefore the limitations of the conventional methods paved the way to the introduction of culture independent molecular methods in the field of microbial analysis and diagnostics.

In recent years, open ended methods like Sanger sequencing, pyro sequencing and deep sequencing of 16s rRNA has allowed the extensive characterisation of the microbial communities in a high-throughput manner. Currently, the Next Generation Sequencing is a landmark in the development of sequencing techniques and has led to significant improvements in the depth and scale of 16srRNA sequencing[11,24].

The high throughput methods have been put to use in the study of the subgingival microbiome in various periodontal conditions. But there is only little information available about the microbial profiles in gingivitis in our native population. So, to address this issue, this study was undertaken to evaluate and compare the microbial profile in the subgingival plaque of patients with gingivitis and in healthy individuals.

In this study, the subgingival plaque samples were collected from 4 healthy individuals and 4 gingivitis patient within 2mm of the sulcus depth using Sterile Gracey curettes. The collected sample was then transferred into the Eppendorf tube containing ionized molecular water and was frozen and stored at -20°C. Genomic DNA was extracted from the 8 subgingival plaque samples of gingivitis and health with the Fast DNA kit and the FastPrep24-5G instrument according to standard protocols. Extracted DNA was then purified and amplified using the 16S V3 (341F) forward and V4 (805R) reverse primer pairs with added Illumina adapter overhang nucleotide sequences.

This study was done using the Illumina sequencing method since it has certain advantages viz. more sequence per run there by having more depth coverage compared to other techniques, sequencing of short 16s rRNA amplicons is sufficient to determine low abundance taxa and the per sequencing cost is comparatively less as stated by **Bartram**[11].

Illumina Solexa was founded by **David Klenerman**, a British chemist in 1998, where single DNA molecules can be sequenced or a clonally amplified template. In 2006, the Solexa Genome Analyzer, the first "short read" sequencing platform, was commercially launched. NGS has markedly accelerated multiple areas of genomics research, enabling experiments that previously were not technically feasible or affordable.

The results of our study showed the presence of 6 phyla, 21 genera and 37 species in the periodontally healthy group and 7 phyla, 32 genera and 59

species in the gingivitis group. There was an overall increase in the number of phyla, genera and species levels in the gingivitis group than in health.

Firmicutes was the most abundant phylum present in both the periodontally healthy and in the gingivitis group comprising about 30% and 24% respectively. The other phyla that were present in almost similar levels in both health and disease were Proteobaceria, Candidatus saccharibacteria, Actinobacteria and Fusobacteria. These findings are in accordance with the earlier studies by **Griffen et al[45] and Park et al[104].** The phyla Firmicutes, Proteobaceria and Actinobacteria comprise of the species that are a part of the early colonisers of the plaque biofilm which includes the gram positive cocci and other organisms.

Synergistetes was the only phylum that was present exclusively in the gingivitis group and is in line with the earlier studies[12,120,149]. In the oral microflora, Synergistetes is recognised as one of the 13 different phyla identified in the Human Oral Microbiome database[31]. The human oral Synergistetes bacteria have been principally divided into one species known as Jonquetella anthropi and two clusters, one of which has been recently characterized as Fretibacterium fastidiosum[149]. Though some information on the presence of synergistetes is available, the association of this phylum to plaque induce gingivitis is still not very clear.

At the genus level, Veillonella was the most abundant group in both periodontal health and gingivitis occupying about 22% and 17% respectively

The genus veillonella comprises of small, anaerobic gram negative cocci which have no flagella, spores or capsule. They have been reported to play a key role as early colonisers to facilitate species succession in a multispecies biofilm community[62].

Actinomyces and Haemophilus were among the abundant genera in health but were also found in lesser abundance in disease which is in accordance with the study by **Colombo et al**[25]. It is also well known that Actinomyces along with Streptocoocus strains are the first or earliest colonisers which bind to the two major salivary components of the pellicle.

A few other genera viz. Saccharibaceria genera incertae sedis, Neisseria, Kingella, Prevotella and Eikenella were the other abundant ones present in similar levels in health and gingivitis group. However, the microbiome of the gingivitis group was more diverse than the healthy group at the genus level which is in line with the fact that ecological succession is not seen as a replacement of the primary species but rather the emergence of new, dominant ones as the biofilm matures[2].

At the genus level, it was also surprising that streptococcus which is a key player in the initiation of the plaque biofilm was not present abundantly in both the groups. The reason for this is not immediately clear. The nature of the lifestyle and dietary habits could have had some influence on this. It may also be possible that the supra-gingival plaque and the occlusal plaque which is caries related contributes to that of cocci in the oral cavity. As our samples

are from the subgingival region, a mild shift in the environmental ecology is to be expected. Similarly, the genus Aneroglobus was present among the abundant ones in the gingivitis group. There are a few reports on the presence of this genus in chronic periodontitis patients associated with rheumatoid arthritis and with smoking. Yet its implication in the pathogenesis of chronic periodontitis is not well understood[20,118].

Dialister are small, anaerobic coccobacilli that grow as small, circular and transparent colonies on Colombia blood agar. It may be difficult to distinguish them from other anaerobic cocci in mixed cultures and often require molecular methods to identify them. The involvement of certain species of Dialister namely D. Pneumosintes and D.invisus in oral diseases such as periodontitis, acute necrotising ulcerative gingivitis and endodontic infections has been well established[31,56]**.** The results of our study are in accordance with the above and its very presence among the most abundant genera in the gingivitis group suggests the change of milieu from a commensal to a dysbiotic one.

Some of the abundant genera present in both the groups belong to the HACEK group which are fastidious, gram negative bacilli and have been frequently isolated from the subgingival environment in various reports[27,88]. Their role in the etiopathogenesis of periodontal disease and their role in systemic involvement have been much reported in literature[27]. Based on this, it is clear that this group of organisms may play a crucial role in the

pathogenesis of periodontal disease. This could be suggestive of the change in the microbial shift which is possibly occurring in the subgingival environment which is not as deep in a periodontal pocket as the samples were harvested from within 2 mm of the sulcus depth. Hence there was no significant change in the microbiome composition between the two groups.

At the species level, Veillonella tobetsuensis and V. Parvula along with Actinomyces odontolyticus and A. Meyeri were among the abundant ones. Veillonella makes up part of the microbial community as the biofilm develops and can form associations with other organisms that can facilitate its establishment of the oral microbial ecosystem[90].

The comparison of abundance of the top 20 species in both periodontal health and disease did not show any statistical significance is suggestive of the fact that there could be no distinct shift in the microbial communities between health and gingivitis. An earlier report by **Abusleme et al**[2] has shown that even in periodontitis a distinct signature microbiome does not exist for sites with bleeding on probing which is a sign of increased inflammation.

It could be speculated that the organisms in the shallow subgingival niche could only be of commensal in nature at this stage and as further changes in the environment become more pronounced there could be possibly be a more radical shift leading to dysbiosis.

The diverse community that makes up for the oral microbiome is known to exist in symbiotic equilibrium and present day lifestyle changes can attribute to the disturbance of this delicate balance. So strategies to re-establish the ecological harmony are vital in the prevention and treatment of periodontal diseases.

The power of newer molecular approaches in defining the composition of the bacterial communities is without a question. But the interpretation of the information generated with the use of these modern technologies should be done with caution as time tested methods of culture, characterisation of the organisms and its role in the etiology of the disease process should be fully understood before meaningful conclusions are drawn.

The therapeutic implications of this microbial analysis are not immediately apparent. However, within the limitations of the study, the results has clearly established as per previous reports that there is no role of for exogenous pathogens other than the normal oral commensals in the development of gingivitis. This study was carried out in a small study population, in a cross sectional manner. Further studies with a larger sample size and of longitudinal in nature is essential to further expand our understanding of the plaque biofilm and its role in the etiopathogenesis of periodontal disease.

Summary and Conclusion

SUMMARY AND CONCLUSION

The goal of this study was to evaluate the sub-gingival microbiome in gingivitis and to compare it with periodontal health by using NGS sequencing. A total of 8 subgingival samples were collected that included 4 periodontally healthy individuals and 4 gingivitis patients. The whole genomic DNA was extracted from the plaque samples and 16s rRNA sequencing was done in a Illumina Solexa sequencer..

A total of 6 phyla, 21 genera and 37 species were found in the health samples and 7 phyla, 32 genera and 59 species were found in the diseased samples. At the phylum level , Synergistetes was absent in the healthy samples but found in the diseased group. At the genus level, organisms belonging to the HACEK group were found in both health and disease, whilc certain genera viz. Dialister and Aneroglobus was found among the abundant ones in the diseased group.

The comparison of the abundance of the top 20 species in both periodontal health and disease revealed no significant difference between them, which could be suggestive of a microbiome that is predominantly commensal in nature at this stage of biofilm. Hence no distinct microbiome was identified in gingivitis. However, the presence of a few genera and species which were found more in the diseased group than in the healthy one also points towards a dysbiotic shift that could possibly be taking place in the

subgingival environment. However, no conclusive evidence could be drawn from this study due to the small sample size and its cross sectional nature.

To conclude, within the limitations of this study, the very presence of this microbial profile could suggest that further environmental changes could lead to dysbiosis and disease progression.

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Annexures

ANNEXURE I- IRB REPORT

RAGAS DENTAL COLLEGE & HOSPITAL

(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
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TO WHOM SO EVER IT MAY CONCERN

Date: 20.12.2017

Place: Chennai

From

The Institutional Review Board

Ragas Dental College & Hospital

Uthandi,

Chennai- 600119.

The dissertation topic titled "**EVALUATION OF SUBGINGIVAL MICROBIOME IN PERIODONTAL HEALTH AND GINGIVITIS USING NEXT GENERATION SEQUENCING TECHNOLOGY**" submitted by **Dr. R.ARVINTH VISHNU** has been approved by the Institutional Ethics Board of Ragas Dental College and Hospital.

DR. N.S.AZHAGARASAN, MDS.,

Member Secretary,

Institutional Ethics Board,

Ragas Dental College & Hospital

Uthandi, Chennai- 600119.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
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RAGAS DENTAL COLLEGE Chennai

ANNEXURE II- PLAGIARISM REPORT

URKUND

Urkund Analysis Result

Analysed Document:	plagiarism.docx (D35097967)
Submitted:	1/29/2018 2:47:00 AM
Submitted By:	ramesh949@gmail.com
Significance:	10 %

Sources included in the report:

plagiarism check.docx (D34299138)

Instances where selected sources appear:

27

ANNEXURE III

CONSENT FORM

I ……S/o, w/o, d/o……
aged about …….years, Hindu/Christian/Muslim …… …….residing at ……do solemnly
And state as follows.

I am the deponent herein; as such I am aware of the facts stated here under

I state that I came to Ragas Dental College and Hospital, Chennai for my treatment for

……
……

I was examined by Dr…….. and I was requested to do the following

1. Full mouth Plaque Score

2. Full mouth bleeding score

3 Measurement of periodontal pocket depth and clinical attachment loss

I was also informed and explained about the collection of plaque during scaling in ……(language) known to me.

I was also informed and explained that the results of the individual test will not be revealed to the public. I give my consent after knowing full consequence of the dissertation/thesis/study and I undertake to cooperate with the doctor for the study.

I also authorise the Doctor to proceed with further treatment or any other suitable alternative method for the study,

I have given voluntary consent to the collection of plaque for approved research.

I am also aware that I am free to withdraw the consent given at any time during the study in writing.

Signature of the patient/Attendant

The patient was explained the procedure by me and has understood the same and with full consent signed in (English/Tamil/Hindi/Telugu?.....) before me

Signature of the Doctor